A dissertation for the degree of Doctor of Philosophy

Polysorbitol Transporter as Novel Vaccine Carrier and Adjuvant Elicits Prolonged Antibody Response

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By

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Under the Guidance of Professor Cheol-Heui Yun

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Abstract

Effective delivery together with the action of adjuvant is essential for enhanced, long lasting and protective efficacy of the vaccine. Thus, the interest to develop such strategy (i.e., delivery tools and adjuvants) for the vaccination is getting a great attention in the public as well as private sectors. In the present study, I evaluated polysorbitol transporter (PST), a polysaccharide-based polymer as a delivery system and an adjuvant in terms of the induction of long-lasting antigen-specific antibody response.

In the first approach, I demonstrated PST for its application as both a potential adjuvant system and polymeric delivery tool for respiratory syncytial virus (RSV) glycoprotein antigen. RSV is one of the most common causes of viral deaths in infants worldwide, yet there are no effective vaccines available. PST is prepared from sorbitol diacrylate and low molecular weight polyethylenimine (PEI). Unlike PEI and the pre-existing experimental mucosal adjuvant, cholera toxin (CT), PST showed no toxicity *in vitro* and *in vivo*. The osmotically active PST formed nano-sized complexes with RGp by simple mixing, retained the antigenic stability and exhibited negative surface charges made them highly effective for the activation of phagocytic cells and enhancement of phagocytosis-mediated uptake. This resulted in an improved cytokine expression and the significant augmentation of RGp-specific antibody production with long-term persistency over 200 days. Interestingly, PST/RGp enhanced phagocytic cell-mediated uptake owing to the osmotic property of PST and negative zeta potential, suggesting that PST could specifically stimulate phagocytic cells for long-lived antigen-specific immune response, where the polysaccharide properties of PST might also play a role in the induction of prolonged memory response.

In the second approach, I have investigated a mechanism on PST-mediated long-lasting antibody response using a model protein, ovalbumin (OVA). The result showed that

PST/OVA targeted mainly APCs and mostly the B cells in mediastinal lymph node (MdLN) after intranasal delivery. As secondary lymphoid organ, this draining lymph node is providing a site for iNKT cell activation at germinal center in response to higher number of CD1d expressed cells specially macrophage, dendritic cells, B cells and neutrophils. Interestingly, a significant number of $CD1d^{+}$ cells were also found OVA positive i.e. antigen specific.

In the third approach, the existence of OVA-specific antibody secreting cells were investigated thoroughly in bone marrow, lung, spleen, MdLN and other lymph nodes Interestingly, PST/OVA system showed persisted antibody response for a long period of time (over 90 days and up to 500 days after the last immunization) The results showed that PST continuously maintained OVA-specific plasma cells in MdLN as well as in bone marrow from days 7 to 90 after the last immunization. OVA-specific germinal center B cells were also appeared in the MdLN from soon after the last immunization throughout the experimental period. On the other hand, the germinal center B cells showed a significantly increased number and characteristic of antigen specific plasma cells at days 60 and 90 after the last immunization in MdLN of PST/OVA-immunized mice upon OVA restimulation *ex vivo*. Furthermore, higher OVA-specific IgG was found in the supernatant in which they were cultured. Interestingly, in MdLN, potential OVA-specific B cells were maintained up to day 90 from the last immunization. It is worthy to mention that, PST exhibited long-term antigen-specific antibody response without showing any polymer-specific antibody response, which is particularly one of the necessary properties for vaccine delivery to improve the efficacy of antigen-specific immunity.

In conclusion, all of the results indicate that PST is a promising adjuvant and functional delivery material for vaccine, devoid of side effects, able to stimulate innate immune

system and most importantly induces the long-lasting antibody response with some unique mechanisms, especially with CD1d-mediated iNKT cell activation.

Keywords: Antibody secreting cells; Antigen Presenting Cells; Cluster of Differentiation 1 (CD1d); Draining lymph node; Long-term antibody response; Natural Killer T cells; Osmotic activity; Ovalbumin; Phagocytosis; Polysorbitol Transporter; Respiratory Syncytial Virus.

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Mesenteric), and v) bone marrow. Total numbers of $TCR\beta^{-}CD11b^{-}CD19^{+}IgD^{-}IgG^{+}$ $GL7^{-}$ $CD138^{+}$ OVA^{+} B cells / organ were analyzed. The PST/OVA group showed OVA-specific populations in MdLN and bone marrow **(Figure 4-3)**. This was further validated in the confocal data. Results in **Figure 4-4** showed that OVA bound on B cells are mostly in MdLN and with a few in the bone marrow, but not in lung and spleen.

Figure 4-4. OVA-specific B cells at days 90 post-last immunization in mice immunized with PST/OVA. Mice were immunized with PBS, PST/OVA and at day 90 several tissue samples like lung, MdLN, Bone marrow and spleen were isolated. Tissue samples were prepared by cryosectioning, staining with Alexa-488 labeled OVA and mounted by DAPI. Confocal microscopy was used to capture sample images. Images are representative of two independent experiments with draining lymph nodes obtained from 2-3 mice per treatment. Scale bars denote 100 μm.

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Chapter I

General Introduction

1-1. Overview of vaccine development

Vaccination has been one of the revolutionary inventions in human history which has provided the power to eliminate numbers of devastating diseases that used to cause millions of deaths worldwide. The appeal on vaccination strategy to control diseases hasn't fade even a little until today although it has been practiced for several hundred years. Due to the tremendous success and effectiveness of vaccination, lifespan has been dramatically increased over the centuries. In ancient time, most of the deaths were occurred because of the fetal infectious diseases of that particular era. In 1750, the lifespan was only about 35 years which increased to 45 to 65 after the middle of 18^{th} and 19^{th} century, respectively. Today the life expectancy in our society is about 80 years and it is further expected to reach to 100 years within few decades [1] (**Figure 1-1**). The main reason for this controlled and steady improvement in life expectancy has been due to the superior control of the infectious diseases with the invention of various effective vaccination strategies over the centuries which prevented early mortality and simultaneously provided longer lifespan to elderly by protecting them from acute and chronic diseases. Some of the biggest success stories of vaccination which provided the crucial benefits of human healths are - the comprehensive eradication of smallpox from the earth, an almost complete protection against polio virus, a more than 95% elimination efficacy against tetanus, pertussis, diphtheria, mumps, measles and rubella and strong effectiveness against Haemophilus influenza type b, pneumonia and hepatitis A & B [2]. Before going further into the deep of explanation on various vaccination systems and its efficacy, here I am providing a brief ride on the history of vaccination and its developments.

Vaccination, the most ground-breaking intervention in medical discovery, is entering to its practice to third century and it is evident that it has prevented about 2.5 million cases each year

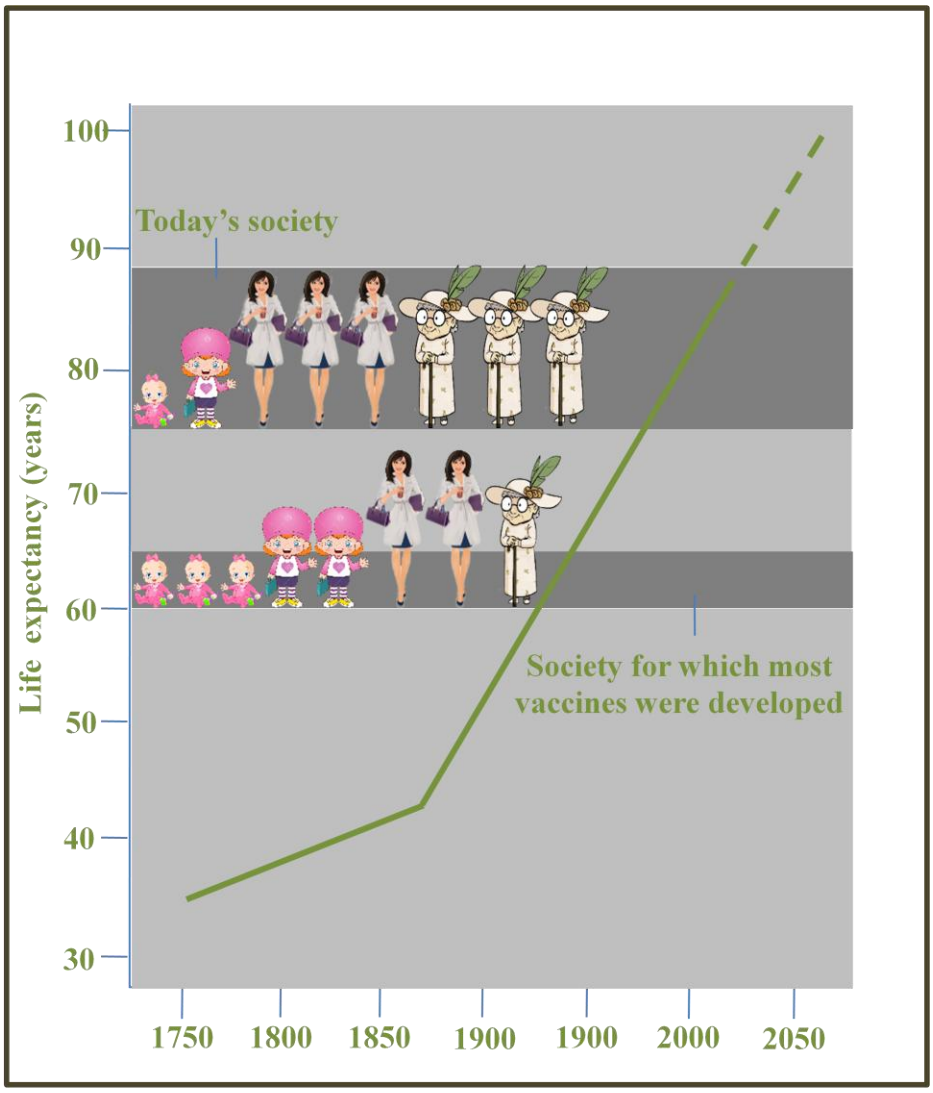

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Figure 1-1. Increase in life expectancy over the time [Ref 1].

world-wide, which is equivalent to an estimation of 7000 deaths in each day [3]. Vaccination technology has a powerful potential to be the most promising tool in clinical application since this field has been leading the way of medical treatment and improving rapidly in each decade with innovative development and new technologies. Over the time in human history, immunity against diseases has been achieved empirically at ancient time and later designed rationally by the researchers to developed vaccine. The first written record of immunity was described in 430 BCE by Thucydides in "The History of the Peloponnesian War" at Greece by describing a contagious disease (plague) that affected thousands of people in Athens [4]. Thucydides described that the people who affected by plague and recovered from it never affected twice and they were not afraid on their own safety against the disease, suggesting the concept of immunity existence at that time. Long after then, the first protective immunization was reported in the 10^{th} century in China against a contagious disease, small pox. In this vaccination strategy, a healthy individual inhaled the dried pustules or inoculated the scratched skin of a mild small pox infected individual in order to be protected from future infection. This technique is called as "variolation' (Latin *'varius'* means spotted) which became very popular in 18^{th} century in Europe when small pox was one of the diseases caused a high rate of deaths (half a million per year) worldwide.

In 1796, Edward Jenner applied the similar 'variolation' technique; however, in this case he used the pustules from cow which is relatively an easy source of pustules to be employed for this vaccination purpose. His immunization method was very effective and could protect from the severity of small pox with minimal side effects. This pioneering discovery by Jenner is considered as the official inauguration of the term 'vaccination' (Latin *vacca* means cow) which was widespread and the small pox was completely eradicated far later in 1979 with a little modification from the original technique proposed by Jenner [5].

The discovery of vaccination by Jenner was a milestone to be followed by the scientists for further improvement in this research field. At that time, however Jenner did not know about the exact action and mechanism of vaccine which was later discovered by Robert Koch and Louis Pasteur where they described that infectious disease is caused by microorganisms. Pasteur attenuated this microorganism by heating, air dry or transferring them to different hosts and tried to use as a vaccine. The first microorganism which was attenuated is the *Pasteurella multocida*; a bacterium causes chicken cholera [6]. In the same way, the first human vaccine was also developed where the rabbit was used as a host to grow rabies virus and was attenuated by air drying before using it to immunize a boy named Josef Meister who was bitten by a rabid dog [7]. Following this discovery, various attenuated vaccines such as anthrax vaccine, Bacille Calmette-Guerin (BCG) against tuberculosis [8], diphtheria [9] and tetanus toxoid [10], influenza and polio [11, 12] vaccine were developed for human use and provided revolutionary health benefits and proof-of-concept on developing effective vaccines until today.

Standing at the twenty-first century, the potentiality of vaccination technology has achieved progressively the high standard over the time and at present we cannot think a better healthy life without the effective contributions of vaccines in our society.

1-2. Barriers and challenges to develop effective vaccine

Vaccine has been playing undoubtedly an extraordinary role to combat against various infectious diseases. Although vaccination system provided eradication and almost nearly completed protection against various diseases, yet there is still the need of new and innovative vaccines, however the progress is slow compared to the demand. Many of the currently available vaccines simply stimulate immune activity and antibody response without a detailed understanding on

their mechanism and their disease protection ability. To manufacture an innovative vaccine, a logical understanding on the underlying mechanism is a must needed criteria to bring it into the market [13]. This is one of the most important challenges to develop effective vaccine. The lack of exactly available animal models that can resemble human system is another barrier to study vaccine efficacy because it is to note that the animal models which are available around us such as pig, mice, rat guinea pig and rabbits may considerably differ the protective immune mechanism compared to the human [14]. For example, the very common animal model mice has the innate immune sensing strategy via toll-like receptors (TLR) which differs from human [15, 16]. Same as in the adaptive immune response, the classes and subclasses of antibodies corresponding to the functions varies greatly between mice and humans since it was described earlier that mice have a single class of IgA with a low level, whereas humans possess two different classes of IgA (IgA1 and IgA2) [17]. It is also to mention that the T-helper (Th) cell differentiation may also be different between mice and humans due to the unclear understanding on the polarization of Th-cell subsets where mice have a good clear picture on this [15, 17]. In many cases it was found that the promising phase II clinical trials fail to translate its efficacy in the human trials [10] which might be due to the irrelevant physiological conditions depending on the age, nutritional state, pre-existing disease state and immunity to the vaccine and vaccine vectors [15-17].

Besides all these physiological barriers, there is also a huge challenge to develop an effective vaccine formulation for human use [18] that can deliver the vaccine to the target site of action and increase its efficacy. Moreover, it should be inexpensive to reach to the market of the mass population. Most of the vaccines are very much susceptible to degradative enzymes and can be eliminated by the body’s immune system before reaching to the actual site of action. Therefore

designing effective delivery system for vaccine is a critical issue. Another crucial factor is the use of appropriate adjuvants to increase vaccine efficacy [19]. Both the delivery system and adjuvants are essential to provide a potent and durable antigen-specific immunity, most importantly to elicit long-lived immunological memory. Next section I am focusing on vaccine delivery systems and adjuvants which are essential to prepare effective vaccine formulation.

1-3. Importance of vaccine delivery systems and adjuvants

During the past few decades, several dramatical and significant swings have been found in immunological practice which is the discovery and application of various novel vaccine delivery systems and adjuvants. The mechanism behind the innovative thinking is the stimulation of the early phase immune response (innate immune responses) and amplification of the antigen-specific immune responses further for the long-term immune activity. Moreover, the identification of specific cell types, receptors and the signaling pathways involved in activation of innate response has provided an assembly of various new targets by the development of novel vaccine delivery systems and adjuvants. Combining these factors, an increasing emphasis has been given in vaccination technology to improve the safety profile, scalability as well as the efficacy particularly against various insidious pathogens (such as respiratory syncytial virus (RSV), human immunodeficiency virus (HIV) and hepatitis C virus (HCV), influenza virus and many others) [20].

In order to induce protective immunity, a vaccine formulation must reach to the target site of action and stimulate immune cells to elicit proper immune response. For targeting vaccine to the appropriate site and increase its efficacy, various delivery system and/or adjuvants have been used over the past years. Delivery systems are basically the particulate systems where vaccines

are loaded inside the particles or make complexes with the delivery materials to formulate vaccine candidate. On the other hand, adjuvants are the compounds which improve the immune efficacy of the vaccine antigen. The adjuvant comes from the Latin word ‘adjuvare’ which means ‘help’ or ‘to enhance’. Thus, adjuvant can be defined in such a way that it is a product which helps vaccine antigens to increase cellular or humoral immune response to protect against diseases. In many cases, antigen itself is very weak immunogenic when adjuvant is a must needed assistive component to intensify immune activity [21]. The concept of ‘delivery system’ and ‘adjuvant’ has been often applied as substitutable terms when we discuss about vaccines. But it is a matter of fact that there is clear distinction between them and their respective roles can be clearly differentiated, especially when a vaccine together with adjuvants gets help to be delivered properly to the action site of the body using a delivery system. Various vaccine delivery systems have been developed for that purposes (for example, viral vectors, electroporation, ultrasound and gene gun (especially these are for DNA vaccine), virus-like particles, liposomal delivery systems, virosomes, emulsion-based delivery systems, polymeric delivery systems (such as microparticles, nanoparticles, micelles, dendrimer-based vaccine carriers, immunostimulatory complexes (ISCOMS). The principal action mode for vaccine delivery system is to endorse antigens uptake by the key antigen-presenting cells (APCs) which are responsible for the induction of immune responses. Moreover, it is important to note that, the potency of these delivery systems can be significantly improved by the addition of a vaccine adjuvant, and/or immune potentiator (**Figure 1-2**). Adjuvants are particularly used in delivery systems to emphasis their impacts onto the APCs, and to minimize effects on non-immune cells. Therefore, both the properties of delivery systems and adjuvants are very essential to design a complete vaccine candidate [20].

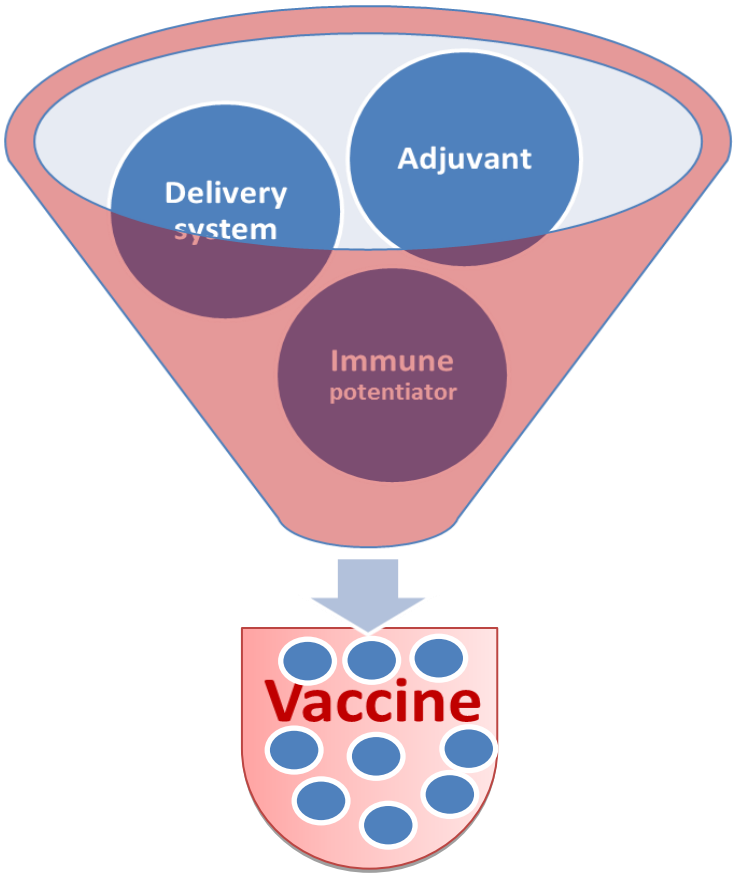

Figure 1-2.Three important components of vaccines to exert their efficacy. Vaccines exhibit more powerful and prolonged antigen-specific immunity when adjuvant is used. As the powerful package components, the delivery systems and the immune potentiators are being used to boost up in vivo vaccine adjuvant efficacy. The delivery systems targets antigens to the appropriate cell types of the innate immune system. On the other hand, the immune potentiators directly activate innate immune cells providing the pro-inflammatory context for antigen recognition. Antigens provide the specific pathogen epitopes necessary to generate long-lived immunological memory. These three components are intrinsic to naturally occurring infections and whole-cell vaccines, whereas they must be combined in vaccine (especially the subunit vaccine) formulations.

1-4. Challenges of available vaccine delivery systems and adjuvants

The successful vaccination against smallpox by Edward Jenner, which is considered the birth of vaccination technology, has dominantly promoted the matter-of-fact that people use the thing what works, regardless the fact why it works. However, an underpinning mechanism should correlate with the theoretical and practical outcomes to develop a safest, best designed and most effective vaccine candidate against a numbers of devastating diseases where vaccines are ineffective at present. The various groundbreaking progresses in basic immunology and material sciences offer to provide greater contributions to establish powerful vaccines by revealing many new targets and mechanisms of immune systems. Although the progress of designing delivery systems and adjuvants has grown with rapid pace, it is frustrating that there are very few systems have been approved for clinical use.

To develop any new and improved adjuvants for vaccines, safety has been the major hurdle, because vaccines should have minimal adverse effects to be approved and acceptable for clinical use. Despite of developing many adjuvants and testing extensively in pre-clinical and clinical settings, it is surprising that only the aluminum salts (also known as ‘alum’) have been successfully licensed so far as vaccine adjuvants for human use in North America [22, 23]. Moreover, the micro-emulsion MF59 (a particulate delivery system), has been marketed in Europe since 1997 to be used in combination with an influenza vaccine [24]. The functional mechanism of MF59 as an adjuvant is to promote the uptake of administered vaccine antigens into APCs (25). MF59 has been tested in a wide range of clinical trials and found to be safe and efficacious (26); however a number of important issues had aroused during its early clinical development. The micro-emulsion, MF59 was used as a delivery system of a potent adjuvant active molecule - the lipidated muramyl tripeptide (MTP-PE) which is a novel synthetic

derivative of mycobacterial cell wall [27]. Although MF59 alone was well-tolerated and exhibited comparable immune-stimulatory activity, the MF59/MTP-PE combination showed too immune-reactogenicity for routine clinical use [27]. MF59 alone subsequently proved its potency and safety, which allowed its successful development of vaccination product [28]. This clinical experience with microemulsions gives light to the fact that there is a need for careful selection of immune potentiators to be included in vaccine formulation. Although MF59 is generally a more potent adjuvant compared to the alum [28], it is not appropriate to use for all vaccines like alum. MF59 is effective to enhance T-cell proliferative responses and induction of antibodies with a variety of antigens [28]; however, Th1-type potent T-cell responses (defined by the production of the cytokine interferon-γ) are not produced by MF59, which is essential to offer defensive immunity against several intracellular pathogens. So, it is clear that an appropriate design and programmed approaches should be employed to develop adjuvant to improve the efficacy of vaccines with good safety profile which are the keys for being approved more vaccine adjuvants in the future [29].

The various particulate delivery systems (such as liposomes, virosomes, emulsions, microparticles, nanoparticles, micelles, dendrimer-based vaccine carriers, immunostimulatory complexes (ISCOMS) and some others) have special features in terms of their dimensions and size which mimic pathogens to make fool of the immune systems to be taken up [30]. The APCs target and uptake these particulate vaccine carriers and process to neutralize as similar mechanism they do with the pathogens. The particulate delivery systems are ingenious enough to release vaccine antigen at the appropriate site inside cells to be presented by the APCs for induction of proper immune response. However, these vaccine delivery systems have various shortcomings such as (i) the inefficiency to stimulate APCs, (ii) insufficient

transfection/targeting activity, (iii) high toxicity, (iv) poor control in immune regulation, (v) lack of long-lasting immune activity and most importantly (vi) many of these show vector-specific antibody responses. All this shortcomings tremendously challenge the effective and rapid development of clinically relevant vaccines and hamper their proper efficacy, addressing the requirement of a new innovative vaccine delivery system which can overcome all the above problems. One of such system that scientist ever produced is the biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) polymer which is most importantly an FDA-approved materials and has been used in many biomedical purposes, including for the controlled release delivery system of a therapeutic protein human growth hormone [31], thus this polymer is an excellent choice as a vaccine delivery system. Kasturi et al. reported PLGA nanoparticles (NPs) as delivery system of a model vaccine OVA along with the use of single or multiple adjuvants [32]. They found that PLGA NPs system was effective for the antigen-specific antibody responses, especially the use of double adjuvants showed the best efficacy compared to that use of single adjuvant. Moreover, the system provided persistence of antibody responses and mechanistically they found that this persistence was occurred in lymph nodes for more than one and half years. This study strongly suggests the importance of using both delivery systems combing with adjuvants and showed the necessity of long-lasting immunity in the development of effective vaccine development. In the recent past, PLGA microparticles were shown to be taken up by APCs *in vivo*, which migrated to the T-cell area of local lymph nodes and differentiated into mature DCs [33]. This work was mainly focused to develop a controlled release system for vaccine antigen and to make a single dose vaccination systems [34]. However, one of the problems associated with this system is the degradation of antigen during encapsulation and release of the vaccine from PLGA microparticles [35].To avoid the problem, antigen was absorbed onto the surface of

microparticles to avoid exposing them to degradation during encapsulation and release. Adsorption was enhanced by using charged surfactants during microparticles preparation, which promoted antigen interaction with the surface of the particles [36]. Consequently, the microparticles were designed to perform as a delivery system to promote the uptake of antigen into APCs, thereby resulting in the induction of potent antibody and T-cell responses in mice [36] and non-human primates [37]. In addition, the adsorption of antigen on microparticles also serves to multimerize the antigens, and so facilitates direct recognition by B-cell antigen receptors. The approach of adsorbing antigens onto charged microparticles has proven sufficiently flexible to allow successful delivery of DNA vaccines, which are adsorbed onto the surface of microparticles. Using this microparticulate system as a delivery system for vaccines resulted in significantly enhanced immune responses in comparison with immunization using naked DNA in mice [38] and in nonhuman primates [39]. The above studies strongly suggest that although PLGA is an approved material for using as a good vaccine delivery system, its efficacy need to be ensured by proper optimizing the formulation procedure.

Another most commonly used material is poly(ethylene glycol) (PEG) which is also FDA approved because of its ability to improve the pharmacokinetic properties of vaccines/drugs, systemic circulation and half-life of vaccines, all of these decrease the doses of vaccination and resulting in the improvement of patient quality of disease state and life [40]. Although PEG reduces the non-specific adsorption of protein, prevent opsonization, flocculation and complement activation [41], it was reported to cause blood clotting and cells clumping which can cause embolism [42]. Recently, it was also reported that PEG can induce antibody response of its own and found that PEG-specific IgM antibodies can be induced by the PEGylated (PEG-modified) NPs which stimulated complement system, leading to the clearance of the NPs [42-

45]. Therefore, the induction of PEG-specific antibody response hampers the efficacy of PEG-based therapeutics.

The above descriptions are the few examples that help to understand that even the FDA-approved delivery materials also have several challenges for successful use in vaccine formulation. There are many other delivery systems also available; however, they have even more shortcomings compared to the approved materials, thus they are struggling to be approved by FDA. Overall, it is important to understand few issues regarding delivery systems of vaccines to come up with a novel formulation idea such as - (i) the delivery vehicles of vaccines need to stimulate immune cells such as APCs to be taken up efficiently before any damage of entrapped vaccines and activate the innate immune system; (ii) the delivery systems should have minimal toxicity to be clinically safe; (iii)They should have the ability to guide immune system (such as APCs) to provide persistent adaptive immunity (antibody response) which is considered as one of the most important criteria for developing a vaccine candidate; (iv) the last but not the least, the delivery systems should not have their own ability to induce immune response which can interfere vaccine efficacy, thus induction of carrier-specific antibody response is not preferred.

1-5. Stimulation of innate immune system and persistence of adaptive immunity: importance, challenge and mechanism

Our immune system has highly diverse and dynamic arrangement in our body which creates protective shield against potentially pathogenic invaders that provide efficient defense against diseases. For greater use of this dynamic immune orientation, vaccines play great role in boosting up and regulating immune activity [46]. However, vaccination system still has

limitations against various diseases and newly emerging pathogens where no vaccines yet exist [47]. In order to design highly effective vaccines, some innovative thinking's are required. Firstly, an appropriate antigen is needed to be selected that can target memory immune responses. Secondly, design of a vaccine formulation that can target and stimulate innate immune system. Activation of innate immune system by vaccine is currently achieving considerable interest and known to have important functions for the advancement of adaptive immune activity [48]. Thirdly, an immune potentiator (improved adjuvant activity) is needed to be designed to stimulate the innate immune system that can trigger the robust and persistent adaptive immune responses which is a vital issue in vaccine development technology. Fourthly, formulate a delivery system to activate appropriate immune cells to target and trigger vaccine formulation to the right site of action [49].

It is well understood that the innate and adaptive immune system are interrelated with each other which was hypothesized by Charles Janeway Jr. [50]. There are two main functions that innate system can develop – (i) a quick reaction (within minutes) against molecular patterns of pathogens and (ii) steady progress (days to weeks) to precisely develop adaptive responses. In one hand, the innate response gives the first line defense, whereas in the other hand the adaptive response is relatively slow [51]. Among many other works, the most important function of innate immunity is to lead the production of inflammatory cytokine responses which in turn lead the activation of antigen-presenting cells (APCs) such as dendritic cells and macrophages. This early response and stimulation of APCs is the foundation of the subsequent development of the specific adaptive immunity [51]. The innate immune system is clever enough to distinguish the pathogens from its self-components using the TLRs which have the ability to detect the pathogen-associated molecular patterns (PAMPs) as the evolutionary conserved signature [54,

55], more specifically the identification of the pattern-recognition receptors (PRRs) [such as tool-like receptor (TLRs)]. A variety of immune cells such as macrophage, neutrophils, dendritic cells, natural killer cells, B-cells and some non-immune cells including epithelial and endothelial cells differentially express the PRRs which activate these cells induce the secretion of cytokines or chemokines and provide maturation and migration of various other cells, hence create adaptive immune responses. It is to note that Janeway's hypothesis on immune responses demonstrated that the APCs (macrophages and dendritic cells) of the innate immune system stimulate and educate some specific lymphocytes of the adaptive immune response by sensing PRRs to start the protective responses [50]. In contrast to dendritic cells and macrophages, lymphocytes identify the distinct antigenic epitopes in a temporally delayed but specific manner via the T-cell receptors (TCRs) or B-cells receptors (BCRs), thus providing specificity and persistent immunological memory in adaptive immune system [52, 53].

B-cell activation is initiated following engagement of the B-cell receptor (BCR) by a specific antigen. B cells can recognize and respond to both soluble and membrane-associated antigen, although recent insights suggest that membrane-associated antigens are more important for B-cell activation *in vivo* [56, 57]. Following antigenic stimulation, B cells can process and present antigen in association with MHC class II molecules, thereby recruiting specific CD4+ T-cell help and stimulating B-cell proliferation and differentiation [58, 59]. Although the precise factors that determine the fate of activated B cells currently remain unclear, B cells can differentiate along two distinct pathways. On the one hand, B cells can differentiate to form extra-follicular plasmablasts that are essential for rapid antibody production and early protective immune responses. On the other hand, activated B cells can enter germinal centres, where they can differentiate into plasma cells, which can secrete high-affinity antibody following affinity

maturation, or memory B cells, which confer long-lasting protection from secondary challenge with antigen [60, 61]. It is now clear that B cells can encounter and respond to antigen through many different mechanisms depending on the nature and size of the antigen itself, as well as on the cellular context and location in which antigen presentation occurs. This provides great versatility in terms of initiating responses that are appropriate to the particular antigen and are therefore the most effective for the protection of the host.

B cell activation, initiated through the B cell receptor (BCR), can be occurred in either a T-cell-dependent (TD) or T-cell-independent (TI) manner [62]. Most long-lived plasma cells (PCs) in the bone marrow are derived from TD responses involving germinal center reactions followed by niches favoring long-term survival. As it usually takes several days for the cognate T cells to help, a prompt TI response provides the first wave of humoral protection by generating short-lived PCs in the extra-follicular foci of the peripheral lymphoid organs such as lymph nodes, spleen, Peyer's patches, and tonsils [63]. Indeed, some TI challenges could also induce long-lived antibody responses [64-66].

It is now widely understood that beneath the protective outer collagenous capsule, a lymph node is divided into three discrete (but non-rigid) regions that are defined by the expression of specific chemokines [67, 68]. Directly beneath the subcapsular sinus is a macrophage-rich sheet that surrounds the B-cell zone that is also termed the cortex. B cells in this region are organized into aggregates, known as follicles, and are the largest population of $IgM^{med}IgD^{hi}CD21^{med}CD23^{hi}$ B cells in the body. Follicles are also rich in radiation-resistant follicular dendritic cells (FDCs) that express high levels of the adhesion molecules vascular cell-adhesion molecule 1 (vCAM1) and intercellular adhesion molecule 1 (ICAM1), as well as complement and Fc receptors [69-72]. FDCs are thought to be of mesenchymal origin and thus form a population of cells that is distinct

from classic DCs [73]. The precise mechanism of FDC development is not yet fully understood, but is known to require the expression of various chemokines receptors and the presence of B cells [60, 74]. Following exposure to antigen, follicles may also contain specialized structures, known as germinal centres, which consist of rapidly proliferating B cells within a network of FDCs. The formation of germinal centres is important during the development of humoral immune responses to T-cell-dependent antigens, as they serve as sites for affinity maturation and the generation of long-lasting memory B cells. A schematic illustration on the overall mechanism of long-lasting immune response is shown in **Figure 1-3**.

1-6. Design of polysorbitol transporter as a novel delivery tool and adjuvant to stimulate APC and persist immunity

It is an exciting time for the functional biomaterials to apply as a delivery tools and if possible as an adjuvants to develop effective vaccine formulation. The highly diverse and tunable properties of biomaterials together with the knowledge of advanced technology and connective interface of a number of discipline such as (but not limited to) material science, immunology, physics, biostatistics, engineering, chemistry, biology, medicine really can boost up the vaccine technology to the next phase of reality [75]. Modulation of the immune system by the engineered materials is an emerging field that is simultaneously approaching with basic immunology. For vaccine therapeutic application, engineering of biologically compatible materials is a hot issue to develop vaccine formulation and deliver antigens through specific intracellular pathways, offering a sustained and controlled way of vaccine delivery to the specific immune cell and to stimulate them for proper immune responses. As stated earlier, design of a material-based adjuvant is also popular concept to design vaccine candidate by mimicking PRRs to make fool of

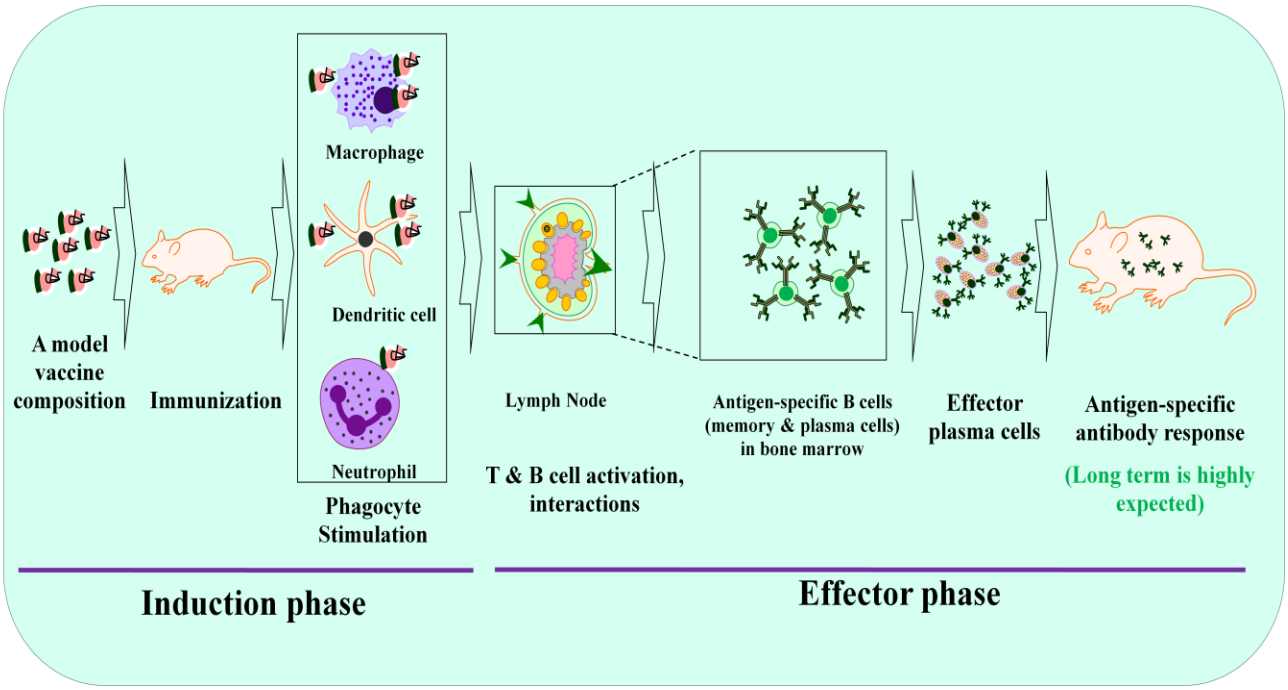

Figure 1-3. A schematic illustration on the overall mechanism of long-lasting immune response.

the immune system and manipulate the innate immune responses. Thus, the materials which can modulate immune system are able to provide new insight into the vaccine technology.

‘The ‘Immuno-bioengineering’ is a word has been used to explain the efforts by the material scientists with engineers and immunologists to design innovative materials as delivery systems for active agents and to better understand the immune system. There are several examples on immune-bioengineering such as the design and engineering of delivery system and adjuvants to activate the innate and adaptive immunity, the engineering of microenvironments to understand the interaction kinetics of APCs and various T-cells, modifications of material surfaces to affect complement activation/neutralization, and last but the not least, stimulation of specialized organs such as lymph nodes and bone marrows by using the engineered biomaterials to aid the long-lasting immune responses. These advanced technologies have tremendous prospects to develop prophylactic and/or therapeutic vaccine strategies to not only prevent infectious diseases but also will be effective to develop immunotherapeutic compounds, against various other diseases such as cancer, diabetes, and various autoimmune diseases. Although, the field is emerging rapidly, yet our understanding on engineered biomaterials to immunology is not well educated, thus need extensive investigations on this highly promising field to bring it into the bed-side clinical application.

In the immune-bioengineering area, the concept of nanotechnology is a revolution and the application of nanotechnology for immunological purpose (such as in the vaccine technology) has been showing tremendous potentials [76]. Nanotechnology has unique objects in terms of physicochemical properties, tunable formulation methods, structural integrity and targeting ability to direct the vaccine formulation specifically to the right immune cells or organs to modulate immune activity. Although the toxicity and vector-specific immune-reactogenicity is

still a concern for developing effective nanomaterials for vaccine delivery, the success on this field will be the next revolution in clinical application against infectious or non-infectious diseases. In this study, I have designed polysorbitol system as a novel biomaterial based on sorbitol molecule and low molecular weight (LMW) cationic polyethylenimine (PEI). Here the polysorbitol transporter (PST) was applied as a delivery tool and potentially as an adjuvant for various vaccines. Most importantly, I did an extensive investigation on PST system throughout various studies to find out the functional mechanisms on how PST interact and modulate the immune system and bring out immunological efficacy for vaccine therapy including the long-term adaptive immunity.

Sorbitol (also known as D-glucitol), an organic osmolyte, is widely produced in plants, particularly in those of the *Rosaceae* family, including apples, cherries, pears, and others. It is produced commercially by reduction of D-glucose or D-glucono-1, 4-lactone and is used extensively in the food industry due to its complete water solubility and lack of any perceptible toxicity [77]. PST possesses polysorbitol backbone which contains many hydroxyl groups. Thus, PST is designed in such a way that it will provide beneficial properties to reduce toxicity because reduction of cytotoxicity was reported by inclusion of hydroxyl groups in polycations [78]. In recent time, PEI has shown to stimulate immune system when used as vaccine carrier [79] and also reported to have adjuvant activity when used for viral subunit glycoprotein antigens [80]. It was demonstrated that PEI showed adjuvant activity owing to its ability to interact with heparan sulfate proteoglycans expressed on antigen presenting cells (APCs) [81]. However, the use of PEI as vaccine adjuvant for clinical use still has the concern of toxicity because of its non-degradable nature, thus suggesting more investigation on how to reduce or eliminate the toxicity

of PEI [80]. If we can do that it might be an excellent vaccine carrier for using in therapeutic purposes.

It is to note that the effective enhancement of cytokine [82, 83] and antibody production [84] can be efficiently augmented by applying osmotic activity onto cells. Thus, it will be highly beneficial if I can formulate a vaccine delivery system with an osmotic active property that could provide the equivalent goals of carrying antigen and intensifying the immune activity. Considering all above factors, I designed the polysorbitol transporter system by combining the sorbitol molecule with LMW PEI by linking them through ester-linkages where sorbitol amount was four-times higher compared to LMW PEI, thus all this factors (ester-linkages, high sorbitol amount with high number of hydroxyl groups, use of LMW PEI with low toxicity by nature) in this design could possibly form PST as a non-toxic material to act as a vaccine delivery tool and adjuvant, thus improving the possibility of using it as a safe carrier for vaccines. Moreover, it is important to mention that the vaccines based on antigenic polysaccharide elicit memory B cells and improve long-lasting antigen-specific antibody response, but the exact mechanism is still unclear [85]. Taken all together, therefore, I designed the polysorbitol transporter and hypothesized that PST could stimulate innate immune system by activating immune cells such as APCs and increase vaccine efficacy by inducing long-term antibody production; however, finding the proper in-depth mechanism is very important which are the prime priorities in all through various chapters in this study.

1-7. Objectives of the studies

There is no doubt that nanotechnology has enormous potential in vaccination technology, however essentially need proper immunological understanding to translate this promising system

to clinical application. Immunology has a well complexed but organized mechanism and it is not easy for a foreign material to evade immune system. Although this field is growing exponentially in a rapid pace in recent time, an entirely non-toxic and efficacious system is yet to be established. To achieve an extra-ordinary together with effective vaccine delivery system and adjuvant, the following properties are must needed – (i) no toxicity, (ii) zero vector-specific immune-reactogenicity, (iii) ability to induce innate immune response (cytokines) by activating immune cells such as APCs and antibody response (iv) and capability to produce immunological memory for inducing long-lasting immunity. The recent advances and advantages in polymeric vaccine delivery and understanding of immunological behavior of materials offer promising solutions to these potential problems. The wide diversity and tunable properties of polymer chemistry allow me to design vaccine carriers as well as effective adjuvants with improved and desirable properties.

The objective of this study is to design such as innovative polymeric system for vaccine delivery which can also be used as an effective adjuvant that can stimulate APCs and induce effective immune responses with long-lasting persistency having minimal toxicity and vector-specific adverse immune reaction. For that purpose, I have synthesized a novel polysorbitol transporter (PST) system synthesized by cross-linking of LMW PEI (MW: 600) with higher amount of sorbitol molecules. **In the first study**, PST was used as a potent delivery system and adjuvant for the respiratory syncytial virus (RSV) glycoprotein (RGp) antigen aiming to establish a vaccine candidate against RSV because yet there is not vaccine available to prevent this viral infection. In vitro and in vivo studies were performed to test the efficacy of PST to deliver RGp and checked the delivery mechanism how PST increase the efficacy to induce long-lasting antibody responses. In **the second study**, I aimed to show more immunological depth of involvement of

any particular functional molecule which potentially provided PST the ability to facilitate the prolonged expression of antibody secreting cells in the induction of long-lasting adaptive immunity. The in-depth immunological mechanism of the adjuvanticity of PST was investigated using a model antigen, OVA. **In the third study**, the aim of this part was to show the how PST utilizes the immune system in its favor to work and stimulate special immune organs and cells for inducing memory responses. Also this chapter showed whether PST has any vector-specific antibody response to establish the proof-of-concept for an ideal vaccine delivery system.

Taking all together, the overall goal was to establish a milestone in the process of development of novel delivery system and adjuvant strategy of vaccine that could be highly beneficial in personalized vaccine therapy in the future.

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Chapter II

Induction of Long-Term Immunity against Respiratory Syncytial Virus Glycoprotein by an Osmotic Polymeric Nanocarrier

2-1. Introduction

Respiratory syncytial virus (RSV) infection is one of the leading causes of viral deaths in infants worldwide [1]. RSV respiratory tract infection causes an epidemic respiratory pathology that provokes thousands of deaths annually due to otitis media and bronchiolitis in infants and young kids [2, 3], and pneumonia in elderly [4]. It is important to note that the repeated reinfection with RSV after recovery from primary infection occurs frequently in infants [5, 6] and adults [7], suggesting an incomplete and short-term protective immunity against the virus. Moreover, RSV reinfection impairs regulatory T cell function and increases the host susceptibility to allergic asthma [8]. Although the control and prevention of RSV infection is a high public health priority worldwide, no effective treatment is available except for an antibody (palivizumab), which has been used as a passive immunoprophylactic treatment [9, 10]. This is however an expensive treatment limited to patients at high risk of severe infection [9-12]. Possible resistance of RSV to palivizumab is also an issue [13]. Motavizumab, another RSV-specific immunoprophylactic antibody, is in clinical trials [1, 14], but not yet approved. Other candidates, such as small interfering RNA (siRNA) [15] and RSV fusion inhibitors [16], are under development, still needing proof of their safety and efficacy.

The most desirable and effective tool for preventing infections, vaccination, is not yet available for RSV. Development of a subunit protein-based RSV vaccine is the preferred strategy, as it would maintain immunogenicity while avoiding the risk of vaccine virulent conversion [17]. RSV glycoprotein (RGp) appears to be a good vaccine candidate, since this molecule plays a key role in RSV pathogenesis and induces neutralizing antibodies and protective immunity [17]. However, the naked protein is readily degraded and loses its antigenic potential when administered *in vivo* [18]. Therefore, design of an effective delivery system is an important

prerequisite to protect the protein from degradation and to maintain its immunogenicity.

Recently, the cationic gene transfection agent polyethylenimine (PEI) has been reported to boost immunogenicity of a DNA vaccine [19]. Also, PEI was shown to act as mucosal adjuvant for viral subunit glycoprotein antigens [20], probably due to its capacity to interact with heparan sulfate proteoglycans expressed on most cells including antigen presenting cells (APCs) [21]. Production of specific antibodies and protection from a lethal infection was superior after a single administration of PEI complexed with influenza hemagglutinin or herpes simplex virus type-2 (HSV-2) glycoprotein D, as compared to other adjuvants [20]. However, the toxicity of PEI is still a matter of concern, and the possibility of using PEI as vaccine adjuvant for human use needs therefore very accurate evaluation [20].

Since osmotic stress of cells is known to effectively enhance cytokine [22, 23] and antibody production [24], vaccine delivery with a polymeric nanocarrier with osmotic activity could achieve the parallel goals of carrying antigen and amplifying the immune response. Recently, I described an osmotically-active polysorbitol carrier based on sorbitol diacrylate (SDA) as a cross-linker and low molecular weight (LMW) PEI, which showed an excellent transfection capacity for DNA [25, 26] and siRNA [27]. Here, I have designed a polysorbitol transporter (PST) as a carrier and adjuvant for RGp. The cross-linking of LMW PEI using SDA could possibly reduce its toxicity by forming degradable ester linkages, thus improving the possibility of using it as a safe carrier for the RSV vaccine. Moreover, since antigenic polysaccharide-based vaccines could elicit memory B cells and improve long-term antigen-specific antibody response [28], it is possible that also the polysorbitol-based vaccine could induce long-term antibody production. In the present study, I hypothesized that, due to the concomitant presence of osmotic activity and immunostimulatory capacity, PST can be an effective vaccine adjuvant and induce a

potent and long-lasting RGp-specific antibody response.

2-2. Materials and Methods

2-2-1. Materials

Branched LMW polyethylenimine (PEI; 600 Da), 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), D-sorbitol, and cytochalasin D (Cyt D) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorbitol diacrylate (SDA) was purchased from Monomer-Polymer & Dajac Labs, Inc. (Trevose, PA, USA). The plasmid encoding the gene of G attachment protein from RSV A2 strain was obtained as previously described [29].

2-2-2. Preparation of RSV-Gp (RGp)

RGp was prepared as previously described [29]. Briefly, the gene corresponding to RGp (amino acid residues 131-230) from RSV A2 strain was amplified from cDNA by PCR and cloned into the pET21d (+) vector (Novagen, Madison, WI, USA). *E. coli* BL21 (DE3) was transformed with the plasmid, bacteria cultured until reaching log phase, and protein expression induced by treatment with 0.5 M isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. Cells were harvested by centrifugation at 6,000 rpm for 10 min and disrupted by sonication on ice. The soluble fraction was applied to a Talon metal affinity column (Clontech, Palo Alto, CA, USA), and the purified protein was dialyzed against 1x phosphate buffer saline (PBS). Endotoxin was removed with Triton X-114, which lowered its level to <5 EU/mg, as measured with *Limulus* amebocyte lysate assay (Lonza, Switzerland). Protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The number of total amino acids,

the molecular weight, and the theoretical isoelectric point are 139, 15491.8 Da and 10.18, respectively.

2-2-3. Synthesis of polysorbitol transporter (PST)

PST was prepared with SDA and LMW PEI by a Michael addition reaction as previously described, with slight modifications [26]. Briefly, SDA and PEI were dissolved separately in DMSO at concentration of 0.836 and 0.209 moles, respectively. The SDA solution was then added drop-wise to PEI (at a feed mole ratio of SDA: PEI of 4:1) with gentle stirring. The reaction mixture was continuously stirred for 24 h at 80°C, then dialyzed (using a Spectra/Pro® membrane of 3500 MW cut-off) against distilled water at 4°C, lyophilized, and stored at -70°C until use.

2-2-4. Characterization of PST

The synthesized PST was characterized by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The molecular weight was assessed by gel permeation chromatography coupled with multiangle laser light scattering (GPC-MALLS) performed with Sodex OHpack SB-803 HQ (Phenomenox Torrels, CA, USA). The chromatography column temperature was maintained at 25°C with a flow rate of 0.5 mL/min, with 0.5 M ammonium acetate used as mobile phase. PST was dissolved in complete culture medium (containing 10% FBS) at various polysorbitol percentages in weight (1, 3, and 5%) and the osmolarity was measured by an automatic cryoscopic osmometer (OSMOMAT_030-D, Gonotec, Germany). A Fourier transform infrared (FTIR) spectrum of PST was measured using a Nicolet Magna 550 Series II spectrometer (Midac, Atlanta, GA).

2-2-5. Physicochemical characterization of PST/RGp complex

2-2-5-1. Morphology, particle size, surface charge and RGp binding efficiency

The ability of PST to complex with RGp was determined by transmission electron microscopy (TEM). Briefly, PST/RGp complexes were prepared at various weight ratios by incubating the components at room temperature (RT) in distilled water (DW) for 30 min or physiological salt solution (pH 7.4) at various time periods (30 min, 8, and 24 h), in a total volume of 2 mL with a final protein concentration of 40 μg/ mL. A single drop of complexes was placed on the copper grid and stained with 1% uranyl acetate solution for 10 seconds followed by several washing with DW. The grid was dried for 10 min and the morphology of PST/RGp complexes was observed by TEM (JEM1010, JEOL, Japan). Particle size and surface charge of PST/RGp complexes were measured by dynamic light scattering spectrophotometer with 90° and 20° scattering angles, respectively, at RT.

To determine the binding efficiency and detect any unconjugated free RGp, the suspension was centrifuged at 17,000 rpm for 25 min at 4^{o}C after making the PST/RGp complexes (weight ratio 5:1) for 30 min at RT. Then the supernatant was collected and the free RGp concentration was measured by BCA protein assay and calculated the RGp binding efficiency in PST/RGp complexes by subtracting the amount of free RGp from the initially used total RGp in the complexes.

2-2-5-2. Circular Dichroism (CD) spectroscopy

To investigate changes in RGp secondary structure after complexing with PST, the PST/RGp complexes (weight ratio 5:1) suspended in distilled water were analyzed by CD spectroscopy (Jasco J-715, Japan) with a quartz cylindrical cell, in comparison with native RGp. Samples were

scanned at the wavelength range between 195 and 260 nm. Values of the CD spectra (mdeg) were plotted against wavelengths to see the differences in RGp structure in native *vs*. complexed form.

2-2-6. Cell lines

The mouse macrophage cell line RAW264.7 and the human adenocarcinomatous type II alveolar epithelial cell line A549 were obtained by American Type Culture Collection, and maintained in Dulbecco's Modified Egles's Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (complete culture medium), at 37°C in humidified air with 5% CO_2. Cells were cultured until subconfluent and subcultured every 2-3 days. Unless otherwise stated, for experimental procedures cells were plated at $1x10^5$/mL/well of 24-well culture plates and grown until about 80% confluent before initiating *in vitro* treatments.

2-2-7. *In vitro* toxicity

Evaluation of *in vitro* toxicity of PST, PEI 25kDa, PST/RGp, and PEI 25kDa/RGp complexes was performed by the MTT assay. Cells were exposed to the compounds for 24, 48, or 72 h in serum-free medium. The number of metabolically active (living) cells was evaluated spectrophotometrically as reduction of the tetrazolium dye MTT to insoluble formazan by cellular enzymes, as previously described [25, 26].

2-2-8. *In vitro* macrophage activation by PST/RGp complexes

RAW264.7 cells were exposed to medium alone or containing PST/RGp complexes, PST, naked

RGp or LPS. Supernatants were collected after 12 h, and stored at -20^{o}C until use. The concentration of TNF-α and IL-10 in the supernatants was determined with commercial ELISA kits (R&D Systems, Minneapolis, USA).

2-2-9. Electron microscopy

Surface morphology was examined by field-emission scanning electron microscopy (FE-SEM) (Carl Zeiss, Germany), while the change of intracellular organelles was analyzed by transmission electron microscopy (TEM; LIBRA 120, Carl Zeiss, Germany).

RAW264.7 cells (1 x 10^5 cells/ well) were seeded on glass coverslips coated with 0.1% gelatin in wells of 24-well culture plates (Sigma Aldrich) grown until about 80% confluent. The cells were then treated with medium, PST, naked RGp, or PST/RGp complexes, and incubated at 37^{o}C for various time periods (for FE-SEM), or at 4°C for 2 h (for TEM). The cells were fixed with Karnovsky's fixative buffer at 4°C for 2 h, washed with 0.05 M sodium cacodylate buffer (pH 7.2), and then fixed again with 1% osmium tetraoxide in 0.05 M sodium cacodylate buffer (pH 7.2), and washed with distilled water. For TEM analysis, cells were further subjected to En bloc staining at 4ºC for 30 min. Samples were dehydrated at RT sequentially using 30, 50, 70, 80, 90, and 100% ethanol for 10 min each. Before FE-SEM analysis, samples were dried using tetramethylsilane (Sigma Aldrich) and mounted on metal subs for gold coating.

For TEM analysis, 100% propylene oxide was added to the specimens for 15 min, followed by sequential exposure to a 1:1 mixture of propylene oxide and Spurr's resin for 2 h, and Spurr's resin alone for 6 h, followed by polymerization at 70^{o}C for 24 h. Finally, specimens were sectioned using an ultra microtome (MT-X; RMC, Tucson, AZ, USA), and finally stained with 2% uranyl acetate and Reynolds lead citrate for 7 min each.

2-2-10. In vitro evaluation of cellular uptake of PST/RGp complexes

For *in vitro* cellular uptake studies, fluorescein isothiocyanate (FITC) (Sigma) was conjugated to the polymer. In brief, PST (4.3 mg/mL) or PEI 25kDa (5 mg/mL) dissolved in sodium bicarbonate buffer (100 mM at pH 8.0) was mixed with a FITC solution (0.5 mg/mL) in the same buffer. The mixture was stirred at RT for 12 h followed by dialysis (using a Spectra/Pro® membrane of 3500 MW cut off) for 2 days in distilled water at 4°C. Finally, the conjugate was lyophilized and stored at -70°C until use. RAW264.7 cells were treated with FITC-PST/RGp complexes (weight ratio 5:1), at different RGp concentrations (3, 5, or 10 μg/mL) in serum-free medium, and incubated at 37°C for 30 min. Untreated and FITC-PST-treated cells were used as controls. Cells were then harvested, washed with PBS and examined by flow cytometry (BD Biosciences, San Jose, CA, USA). Uptake of fluorescent complexes was determined by calculating the geometric mean fluorescence intensity (MFI) of the FITC-positive cells. At least 10,000 cells were acquired, and the experiment was performed in triplicate.

For inhibition of phagocytosis, RAW264.7 cells, seeded at an initial density of 4 x 10^5 cells/well in a 6-well culture plates and cultured until approximately 80% confluent, were pre-treated with increasing concentrations of the phagocytosis inhibitor cytochalasin D (Cyt D) in serum-free medium for 1 h. Cells were then exposed to 10 μg/mL of FITC-PST/RGp complexes (weight ratio 5:1) or PST/FITC-albumin complexes in serum-free medium for additional 30 min. Uptake was determined cytofluorimetrically as described above. Inhibition of phagocytosis was calculated in comparison with control cells in the absence of Cyt D.

To compare PST uptake by RAW264.7 and A549 cells, both cell types were cultured separately on gelatin-coated glass coverslips as described above, treated with Cyt D (1.5 μg/mL) for 1 h, and exposed to FITC-PST/RGp or FITC-PEI 25kDa/RGp complexes for 30 min. Cells were

then washed with PBS, fixed first with 4% paraformaldehyde for 10 min at 37^{o}C and then with a mixture of 100% methanol and acetone (1:1) for 10 min at -20^{o}C. Coverslips were washed twice with PBS, blocked with 10% FBS for 1 h at 37^{o}C, then washed and mounted with DAPI containing fluoroshield solution. After sealing with cover glasses (Marienfeld, Germany), slides were observed using image restoration microscopy (IRM, Delta Vision RT, WA 98027, USA) at RT. At least 1,000 cells per sample were evaluated.

2-2-11. Animals

Six week-old female BALB/c and C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained throughout the study in a controlled environment: 24 ± 2°C, 50 ± 10% relative humidity and a 12 h light/dark cycle. All procedures were performed in accordance with rules and regulations of Institute of Laboratory Animal Resources, Seoul National University (IACUC No.: SNU-120308-4).

2-2-12. *In vivo* toxicity and inflammation study

C57BL/6 mice were administered intranasally with PST (100 and 250 μg/mouse in PBS) in a volume of 10 μL. Cholera toxin (CT; 2 or 10 μg/mL in PBS; intranasally) was used as control. Body weight was measured before, and 2 and 4 days after the administration. At day 4, mice were sacrificed, and lungs perfused with PBS containing 1% heparin were weighed and photographed.

BALB/c and C57BL/6 mice were inoculated with different concentrations of PST (100 and 250 μg) and CT (2 μg) in 50 μL to the each of the two hind paws. After 24 h, the size of paw edema was measured with a slide calipers.

2-2-13. In vivo immunization and determination of antigen-specific antibody response

C57BL/6 mice (n=3) were immunized intranasally by instilling 20 µg of RGp alone or within PST/RGp complexes (weight ratio 5:1) suspended in PBS. Positive control mice received 20 µg of RGp mixed with 2 µg of CT. Immunization was performed three times at two-week interval. Blood samples were collected retro-orbitally to determine antigen-specific antibody levels in serum on days 14, 28, 35, 77, 119 and 203 after the first immunization.

To measure the presence of antigen-specific antibodies in serum, ELISA plates were coated with RGp (5 µg/mL diluted in PBS, 100 µL/well incubated overnight at 4°C), blocked with 1% BSA in PBS, washed, and then incubated with serial dilutions of sera in blocking buffer (200 µL/well) for 1 h at 37°C. After washing, antigen-bound antibodies were detected with goat anti-mouse antibodies conjugated with HRP (Southern Biotech, Birmingham, Alabama, USA) followed by 3, 3´, 5, 5´- tetramethylbenzidine dihydrochloride (TMB), as HRP substrate (Sigma Aldrich). After 20 min, the reaction was stopped using 1N H_2SO_4 and the reacted substrate was measured spectrophotometrically at 450 nm using VERSAmax tunable microplate reader (Sunnyvale, USA). Antibody concentrations were determined after normalizing the readings with the plate background using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

2-2-14. Statistical analysis

The data are reported as mean ± SD, and statistically significant differences between samples were determined by the one way ANOVA, considering as significant P values <0.05. Flow cytometric data and IRM photographs were quantified with the FlowJo (New Jersey, USA) and ImageJ (NIH, USA) softwares, respectively. Data were analyzed and graphically presented using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

2-3. Results and Discussion

2-3-1. PST is successfully synthesized and characterized

PST was successfully synthesized from SDA and LMW PEI (MW: 600 Da) by a Michael addition reaction. The PST synthesis reaction scheme is shown in **Figure 2-1A**, where the different functional properties of the compound are depicted, *i.e.*, the degradable ester linkage, the proton sponge active part, and the osmotically-active transporter backbone. The composition and the final MW of the synthesized PST were determined by ^{1}H NMR spectroscopy (**Figure 2-1B**) and GPC (**Table 2-1**), respectively. As expected, PST was completely water soluble because of the hydrophilic nature of its polysorbitol part. The osmolarity of PST was increased in a polysorbitol dose-dependent manner (**Table 2-2**) as a similar tendency to that of the prime osmolyte D-sorbitol.

Degradability is one of the essential properties in developing a safe and efficient polymeric vaccine carrier for clinical use. PEI, despite being a potent adjuvant, is reportedly highly toxic *in vitro* and *in vivo*, possibly due to its lack of degradability [12, 17]. The ^{1}H NMR spectra of SDA (pre-reaction) and PST (post-reaction) confirmed the successful reaction. The peak for the acrylate groups of SDA was found in 5.8 to 6.5 ppm values (indicated by the lower framed inset), whereas those peaks were disappeared (indicated by the upper framed inset) when SDA was reacted with PEI, confirming the reaction by formation of ester linkages in PST (**Figure 2-1C**). PST with degradable ester linkages was susceptible to hydrolysis, resulting in LMW byproducts

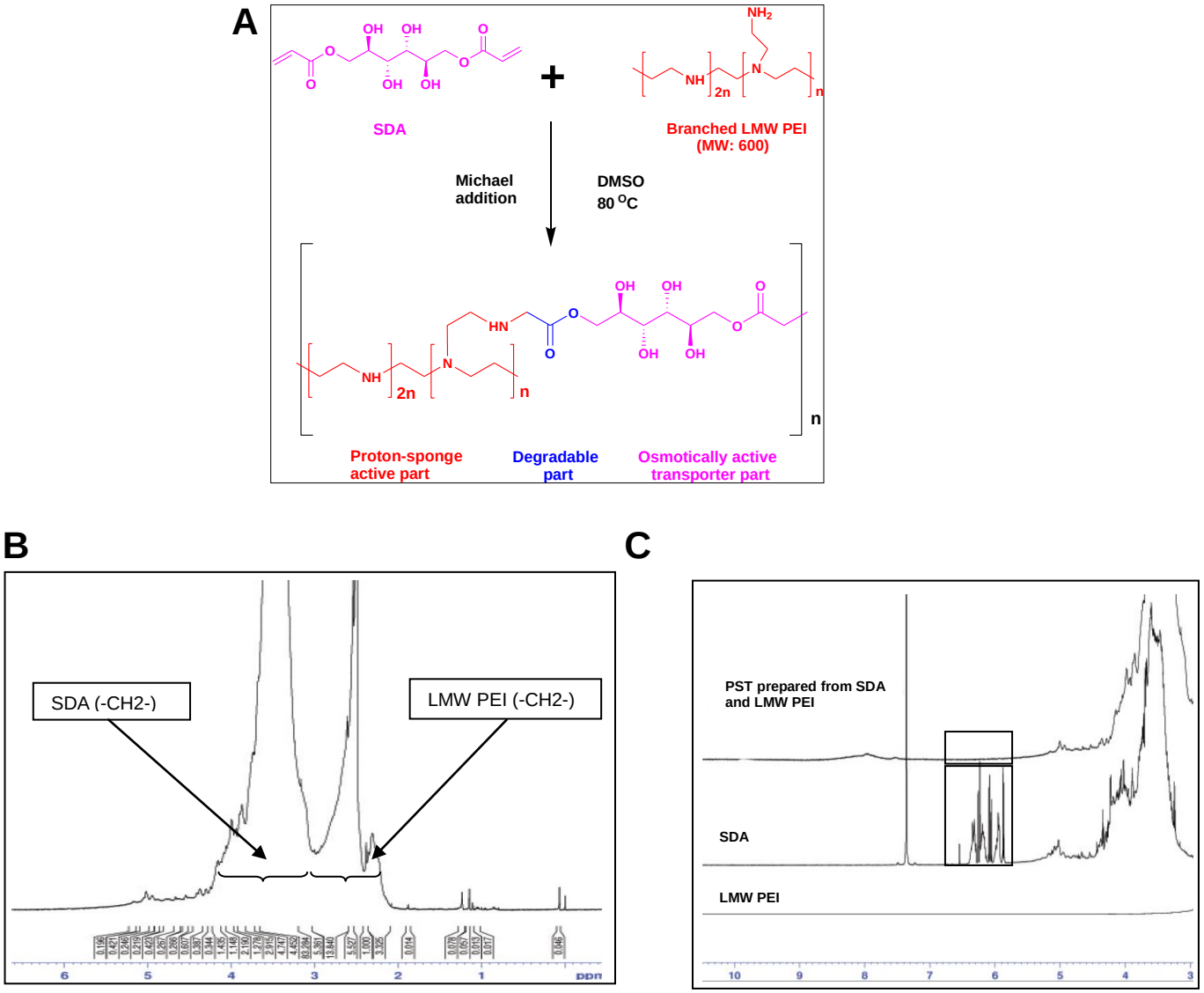


Figure 2-1. Physicochemical characterization of polysorbitol transporter (PST). (A) Reaction scheme of PST prepared from SDA and branched LMW PEI 600 (Feed mole ratio of 4:1 for SDA: PEI). (B) The ^{1}H NMR spectrum of PST. (C) Comparison of the ^{1}H NMR spectra of branched LMW PEI, SDA, and synthesized PST. The lower and upper framed insets indicate the peak for the protons of acrylate groups of SDA and PST before and after SDA was reacted with PEI, respectively.

Table 2-1. Characterization of PST synthesized from SDA and branched LMW PEI

Molecular weight (Da) of		Feed ratio of SDA:PEI (mol: mol)	Composition of SDA (mol %)[a]	Composition of PEI (mol %)[a]	Number average molecular weight (Mn)[b]	Weight average molecular weight (Mw)[b]	Polydis-persity index
Sorbitol diacrylate (SDA)	Poly-ethylenimine (PEI)						
290.27	600	4:1	85.45	14.55	4,900	9,180	1.874

[a]determined by ^{1}H NMR
[b]determined by GPC

Table 2-2. Osmolarity of PST

Molecule	Sorbitol/polysorbitol (wt %)	Osmolarity (mOsm/L)
D-sorbitol	1	335
	3	476
	5	568
PST	1	274
	3	294
	5	314

(acids and alcohols) [17], thus ensuring the degradability that should reduce the toxicity of the transporter [17]. Importantly, PST maintains the osmotic activity that would potentially enhance phagocytosis [22, 23], cytokine production [14, 15] and antibody response [16, 24].

2-3-2. PST stably complexed RGp with suitable particle sizes and surface charges

The morphology, size and surface charge of polymeric vaccine carriers are important parameters for their effectiveness, toxicity, biological fate (cellular uptake, biodistribution), and efficacy. PST/RGp complexes were prepared (at weight ratios of 0.5, 1 and 5) and observed by TEM. It was found that PST stably complexed with RGp at the ratio of 5:1, through hydrogen bonds and ionic interactions, forming uniformly distributed compact particles of spherical shape (**Figure 2-2A**). At lower ratios (0.5 or 1), PST could not form stable complexes with RGp (data not shown). Therefore, PST/RGp complexes formed with a ratio of 5 were used throughout the following studies of characterization and functional efficacy.

Diffusion of exogenous agents delivered to the mucosa depends on the mesh-pore size of the mucus layer [25]. EM analysis reported the nasal and lung mucus mesh spacing ranging between 20 and 200 nm [26]. Since the average size of PST/RGp complexes is 61.5 nm (**Figure 2-2B** showed size distributions), this suggests that the PST/RGp nanoparticles may be able to diffuse through the nasal mucus being therefore suitable for intranasal vaccine delivery.

Surface charge is an important parameter that affects phagocytosis of particles by professional phagocytes such as macrophages [27]. It was reported that phagocytosis of both negatively and positively charged particles increases with the increase of the absolute value of zeta potential [28]. Moreover, phagocytosis of neutral particles was found to be reduced in comparison with that of charged particles [29]. In general, the higher the surface charges the more the

hydrophilicity, which created a stealth effect with consequent reduced phagocytosis by macrophages [27]. Bhattacharjee et al. described that with amine-containing positively charged particles the phagocytic index was reduced, whereas it was increased in a dose-dependent manner with negatively charged particles [30]. The average surface charge of PST/RGp nanoparticles was -14.2±3.5 mV, suggesting no stealth effect and efficacious phagocytosis of the negatively charged PST/RGp nanoparticles by macrophages. Surface charge is also reported as an important factor for their cytotoxicity, with positively charged particles showing increased toxicity due to induction of intracellular reactive oxygen species (ROS) production and to reduction of mitochondrial metabolic activity [30]. Thus, the negatively charged PST/RGp nanoparticles are expected to be a non-toxic vaccine/adjuvant system with improved uptake by phagocytes, which would facilitate antigen presentation and consequent establishment of protective immunity.

2-3-3. Secondary structure of RGp is not affected in complexation with PST

Next, I examined the structural integrity of the protein when complexed with PST [31]. After complexing with PST, the RGp secondary structure was examined by CD and compared to that of uncomplexed RGp. No significant change in the secondary structure of RGp was detectable after complexing with PST (**Figure 2-2C**).

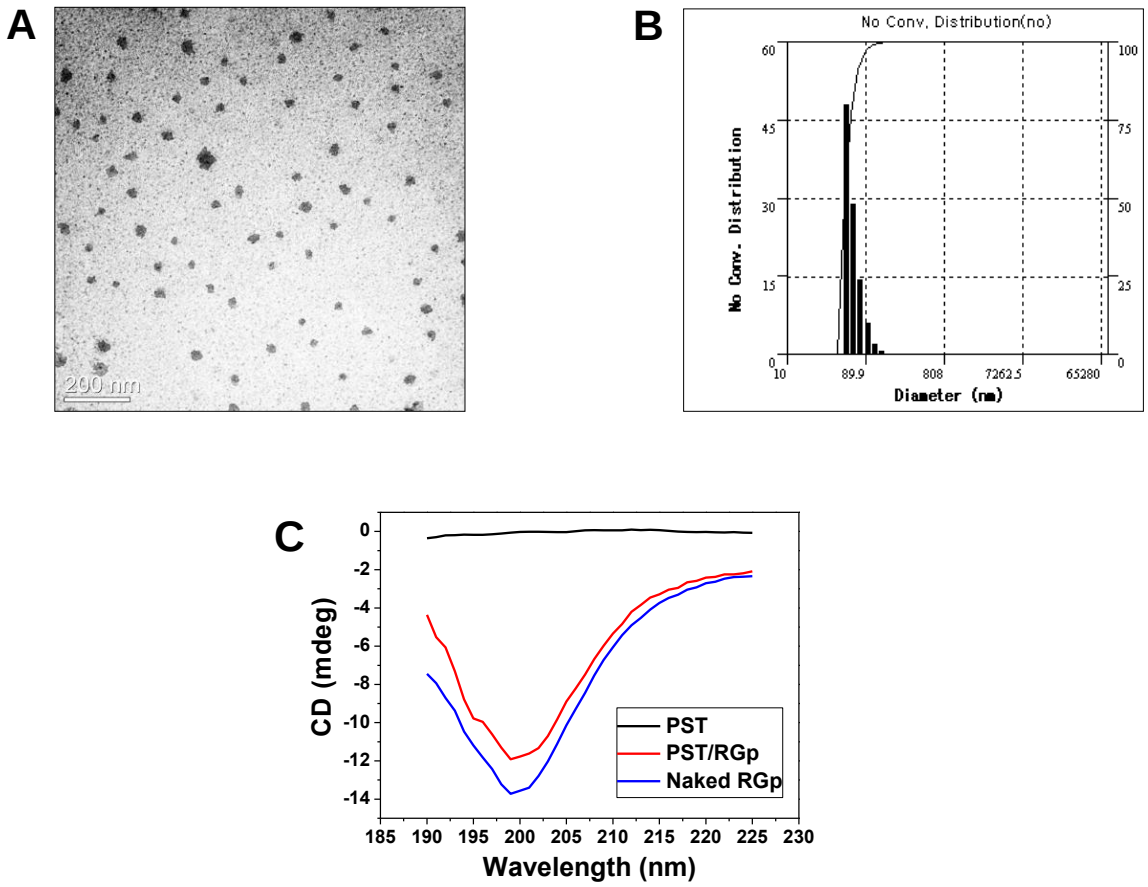


Figure 2-2. Physicochemical characterization of PST/RGp complexes. (A) PST and RGp were mixed at weight ratio of 5:1 and incubated at room temperature for 30 min for complex formation. The images of PST/RGp complexes were captured by TEM (bar denotes 1 μm). (B) The particle size distributions of PST/RGp complexes at weight ratio of 5:1 measured by DLS and was found 61.5 nm with polydispersity index of 1.390E ± 000. The zeta potential of PST/RGp complexes was also determined by DLS at weight ratio of 5:1 and was found -14.2 ± 3.5 mV (mean ± SD). (C) The structure of complexed RGp was examined by CD spectroscopy and compared with native RGp. The wavelength ranges used were between 195 - 260 nm.

2-3-4. PST is safe carrier for RGp *in vitro* and *in vivo*

CT, an effective experimental mucosal adjuvant, is not likely to achieve license for human use [32] because of its toxicity [33]. On the other hand, PEI is widely used as gene carrier and it was reported as a potent mucosal adjuvant for viral glycoprotein antigens [12]. However, also in the case of PEI toxicity should be accurately tested, in addition to effective dose optimization [12, 34]. Here, I investigated the toxicity of PST for the mouse macrophage RAW264.7 cells, in comparison to that of PEI 25 kDa. It was found that cell viability was not affected by PST even at the highest concentration tested (50 μg/mL) for at least 72 h, in contrast to the significant toxicity of PEI 25kDa that reduced cell viability by near or over 80% at 50 μg/mL (**Figure 2-3 A**). Toxicity of the 5:1 PST/RGp complexes was tested at increasing RGp concentrations (0, 1, 2, 6, 10 and 20 μg/mL). The PST/RGp complexes was found to have a very limited toxicity even at the high concentrations (maximum 20%) as opposed to the significant toxicity (more than 90 %) of the PEI 25kDa/RGp complexes (**Figure 2-3B**). PEI is highly toxic because of its non-degradable nature, which may be the cause of necrotic cell death (immediate) or apoptosis (delayed) [34]. On the other hand, PST appeared to be remarkably safe even at a very high concentration, which may be attributed to its degradable ester linkages and presence of many hydroxyl groups [17].

The toxicity of PST was further tested *in vivo* in mice, upon administration via the intranasal route, since this would be the optimal immunization route in future vaccines against RSV, which is a lung infection. No variation in the body weight was observed in mice treated with PST at high doses (100 and 250 μg/mouse) until day 4, similar to PBS-treated group. On the other hand, mice treated with the standard adjuvant CT at two different doses (2 and 10 μg/mouse) lost their body weight significantly in a time-dependent manner (**Figure 2-3C**). PST did not cause any

significant damage to lungs even at the dose of 250μg/mouse (**Figure2-3D**). Lung weight was not altered in PST-treated mice as compared to control PBS-treated mice, whereas the size and weight of lungs were increased in CT-treated mice (**Figure 2-3E**). This indicates that PST does not cause adverse effects on lungs when delivered intranasally.

As additional evidence of the lack of toxicity of PST, edema formation after inoculation into the mouse paw was measured. As opposed to CT, which induced a significant local edema (4x the size of the control paw injected with PBS), no significant increase was detected with PST, again suggesting that PST is safe (**Figure 2-3 F**).

2-3-5. Immune modulation by PST/RGp complexes in macrophage

To investigate the capacity of PST/RGp to stimulate innate immunity, *i.e.*, to have adjuvant capacity, we have examined the *in vitro* production of TNF-α and IL-10 in RAW264.7 cells treated with PST/RGp. Production of soluble TNF-α was increased in RGp dose-dependent manner in cells treated with PST/RGp (**Figure 2-4A**), showing that RGp maintained its immunostimulatory activity after complexation with PST. On the other hand, neither naked RGp nor PST-complexed RGp induced IL-10 secretion (**Figure 2-4B**), in agreement with the notion that RSV suppresses the production of early immunostimulatory cytokines through induction of IL-10 [35]. This finding suggests that the PST/RGp complex, not retaining the IL-10-inducing capacity of RSV, could represent an effective immunizing complex.

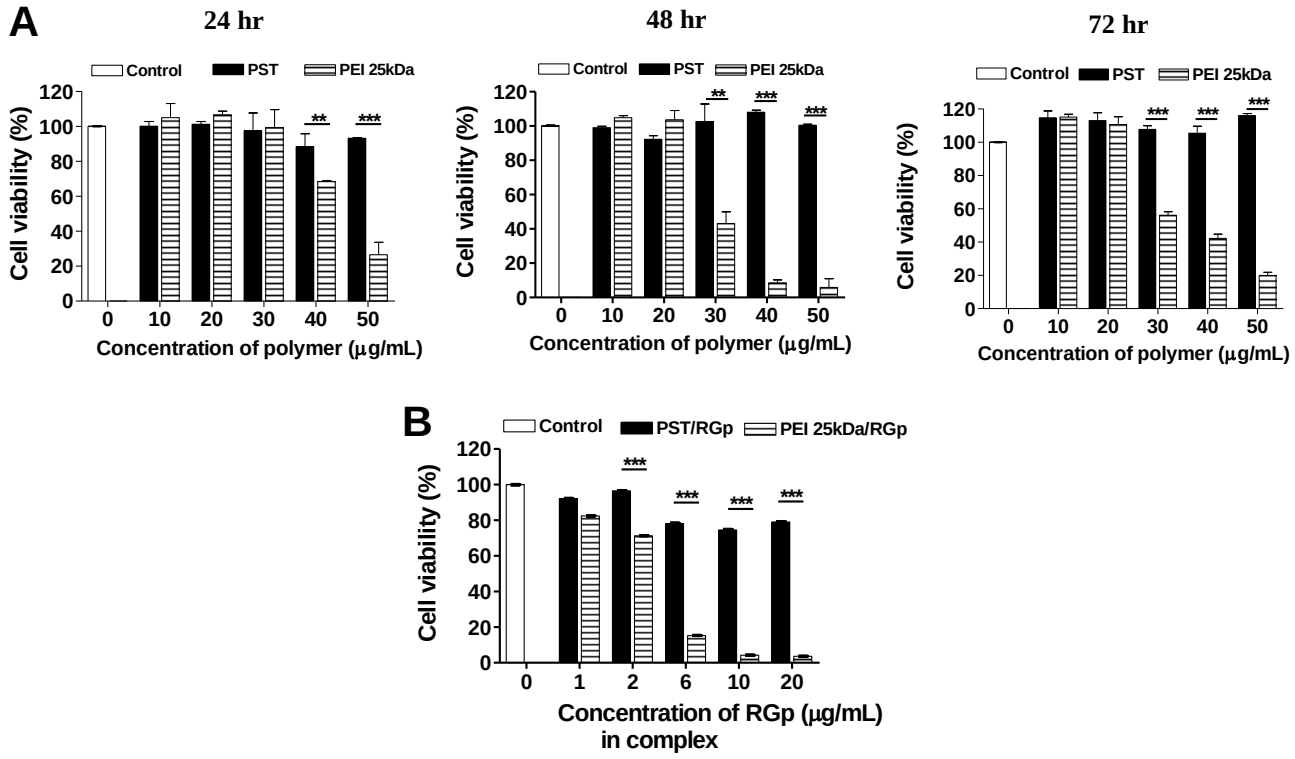

A
24 hr
48 hr
72 hr
Control
PST
PEI 25kDa
Cell viability (%)
Concentration of polymer (μg/mL)
B
PST/RGp
PEI 25kDa/RGp
Concentration of RGp (μg/mL) in complex
C
Day 0
Day 2
Day 4
Body weight (%)
PBS
PST (μg)
CT (μg)
D
PST (100 μg)
PST (250 μg)
CT (2 μg)
CT (10 μg)
E
Lung weight (normalized to PBS)
F
BALB/c
C57/BL6
Paw edema size (mm)
(μg/paw)

Figure 2-3. Toxicity of PST and PST/RGp complexes *in vitro* and *in vivo*. The *in vitro* viability of RAW264.7 cells was tested by the MTT assay upon incubation for 24 h, 48 h and 72 h with (A) increasing concentrations of PST and (B) increasing concentrations of the PST/RGp complexes (weight ratio 5:1) (RGp from 0 to 20 μg/mL). PEI 25kDa and PEI 25 kDa/RGp complexes (weight ratio 5:1) were used as controls, respectively. To test *in vivo* toxicity, mice were administered intranasally with PST (100 and 250 μg/mouse), with cholera toxin (CT; 2 and 10 μg/mouse), and with PBS as control. (C) Body weight measured at days 0, 2 and 4. (D) Images of perfused lungs at day 4. (E) Weight of perfused lungs at day 4. (F) Paw edema development in BALB/c and C57BL/6 mice. Mice were injected with PBS alone or containing PST or cholera toxin (CT). Paw edema size was measured with digital 150 mm caliper and expressed as mean ± SD of 4 values (both paws/mouse, 2 mice/group). $^{**}P < 0.01$; $^{***}P < 0.001$, by one-way ANOVA.

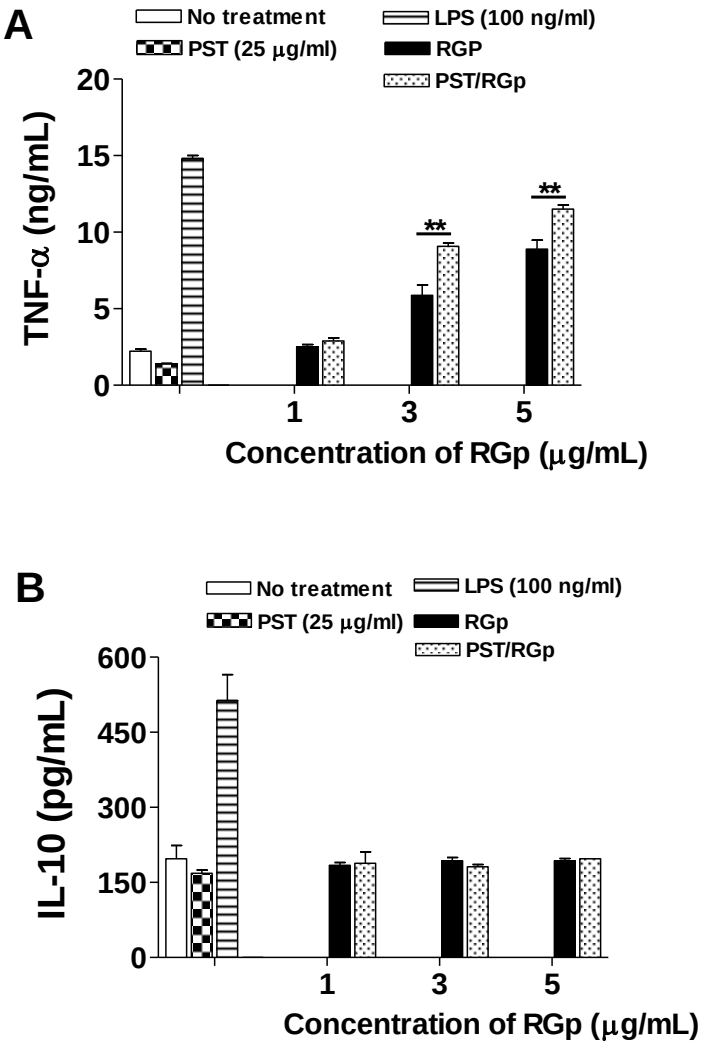
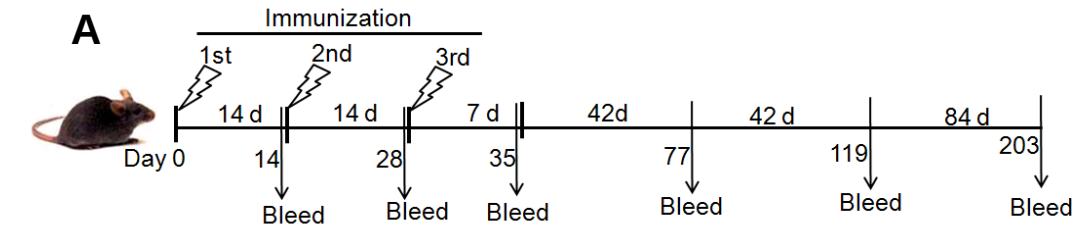


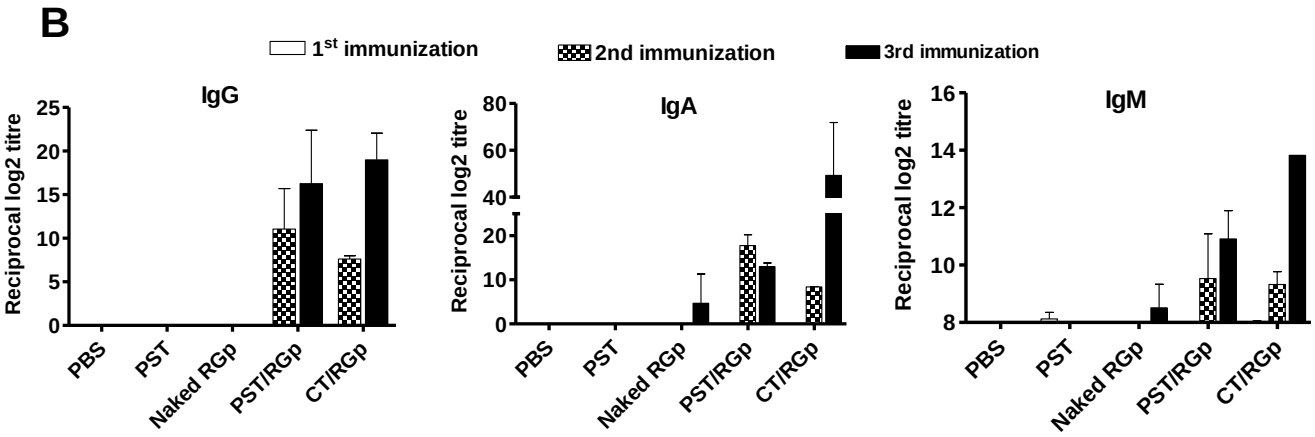
Figure 2-4. Immune modulation by PST/RGp complexes in macrophage to evaluate the functional effects of PST/RGp *in vitro*. To check immune stimulating activity by PST/RGp, RAW264.7 cells were treated with increasing concentrations of the PST/RGp complexes (weight ratio 5:1), supernatants were collected at 12 h, and the production of (A) TNF-α and (B) IL-10 was measured. All data are presented as mean of triplicate measurements ± SD. ***P*<0.01; ****P*<0.001, by one-way ANOVA.

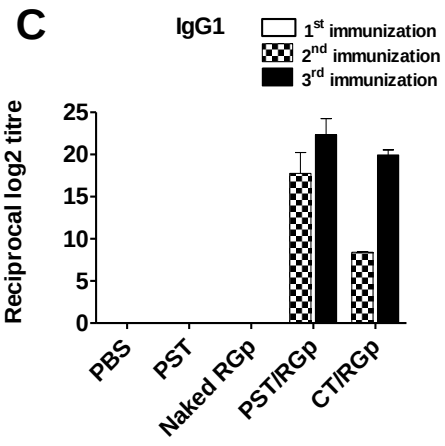
2-3-6. PST/RGp complexes showed enhanced antigen-specific antibody responses with long-term persistency

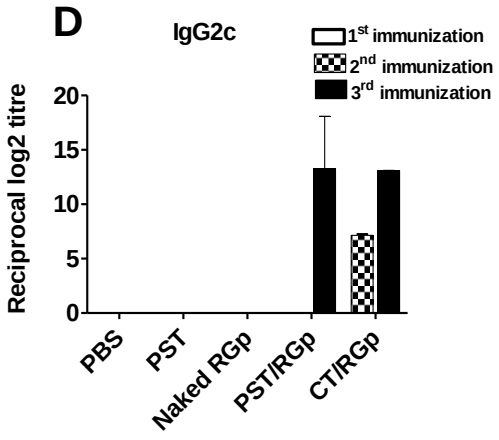
Next, we immunized C57BL/6 mice intranasally with the PST/RGp complexes (**Figure 2-5A**) and compared the RGp-specific serum antibody production to that induced by unconjugated RGp or by CT/RGp. Anti-RGp IgG, IgA and IgM titers in serum were significantly induced by two and three immunizations both in the PST/RGp group as compared to RGp alone. After the third immunization, the specific IgG titer in the PST/RGp group was comparable to that in the positive control group CT/RGp (**Figure 2-5B**). Both IgG subtypes IgG1 and IgG2c were induced by three immunizations with PST/RGp, similarly to CT/RGp (**Figure 2-5C, D**).

To examine whether PST/RGp can induce a persistent antibody response, the antigen-specific serum IgG and IgA titers were measured 77, 119 and 203 days after the first intranasal immunization. Both IgG (**Figure 2-5E**) and IgA titers (**Figure 2-5F**) were persistently high in mice immunized with PST/RGp, showing that PST/RGp can induce a long-term antibody response. Induction of long-term antigen-specific IgG responses by polysaccharidic vaccines has been reported in other instances [36]. A possible mechanism by which long-lasting antibody response to a polysaccharide vaccine can be induced is the stimulation of a T-independent type II immune response, generating memory B-cells [37]. Another possibility is that the continuous and long-lasting formation of antigen-specific short-lived plasmablasts in spleen and a latent population of antibody-secreting cells in bone marrow could cause a long-term maintenance of antibody responses against polysaccharidic antigens [38]. It should be also noted that antibody responses can augment due to osmotic stress [16, 24]. Eventually, it is known that PEI is an effective mucosal adjuvant that can induce antibody responses against viral glycoprotein antigens [12].


A
Immunization
1st
2nd
3rd
14 d
14 d
7 d
42d
42 d
84 d
Day 0
14
28
35
77
119
203
Bleed
Bleed
Bleed
Bleed
Bleed
Bleed


B
1st immunization
2nd immunization
3rd immunization
IgG
IgA
IgM
Reciprocal log2 titre
PBS
PST
Naked RGp
PST/RGp
CT/RGp


C
IgG1
1st immunization
2nd immunization
3rd immunization
Reciprocal log2 titre
PBS
PST
Naked RGp
PST/RGp
CT/RGp


D
IgG2c
1st immunization
2nd immunization
3rd immunization
Reciprocal log2 titre
PBS
PST
Naked RGp
PST/RGp
CT/RGp

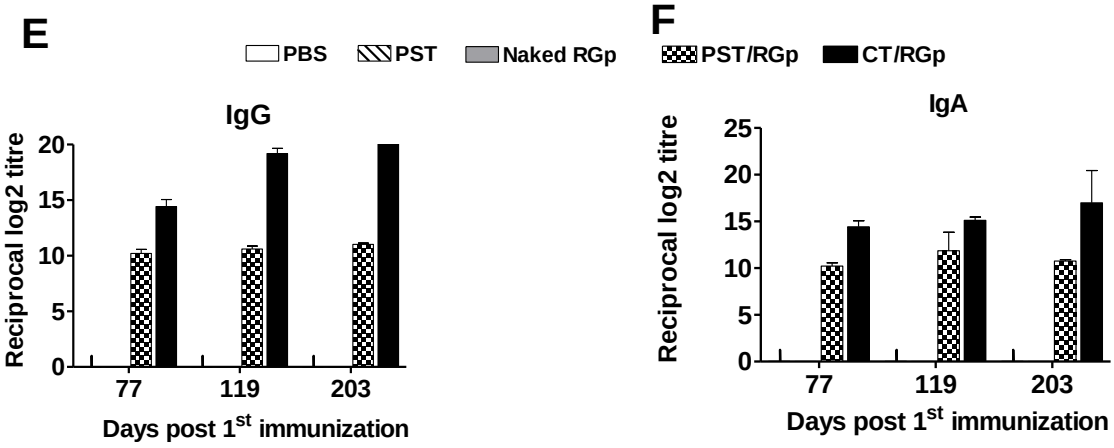

E
F
PBS
PST
Naked RGp
PST/RGp
CT/RGp
IgG
IgA
Reciprocal log2 titre
77
119
203
Days post 1st immunization

Figure 2-5. Antigen-specific antibody responses in mice treated with PST/RGp complexes (at weight ratio of 5:1) or CT/RGp formulation. (A) Scheme of immunization and blood collection. (B) Levels of antigen-specific IgG, IgA and IgM were measured 14 days after the 1stimmunization, 14 days after the 2nd immunization, and 7 days after the 3rd immunization. Antigen-specific antibody production i.e. IgG subtypes in C57BL/6 mice immunized intranasally with PST/RGp. (C) IgG1 and (D) IgG2c titers were measured 14 days after the 1st and 2nd immunization and 7 days after the 3rd immunization. Long-term and persistent (E) IgG and (F) IgA titers were measured at 77, 119 and 203 days after the 1st immunization (mean ± SD, n=3).

Thus, we anticipate that PST/RGp induced a significant and long-lasting antigen-specific antibody response due to the presence in PST of (i) an osmotically active polysorbitol chain that provides immune amplification by osmotic stress, (ii) a synthetic polysaccharide (the same polysorbitol chain) that may contribute to inducing B cell memory and/or continuous activation of plasmablasts, and (iii) an additional mucosal adjuvant activity due to the PEI backbone.

2-3-7. Mechanism of antibody responses by PST/RGp system

2-3-7-1. Surface morphology confers macrophage activation

Morphological plasticity is a known sign of macrophage activation [39]. Macrophages treated with RGp showed finger-like projections already 5 min post-treatment, while PST/RGp complexes started to induce activation-dependent surface changes after 30 min. After 60 min, the surface changes indicating macrophage activation were almost identical with treatment by either naked RGp or PST/RGp complexes (**Figure 2-6**). However, the size of the PST-treated macrophages was increased compared to that of untreated control cells, presumably due to the

osmotic effect of PST resulting in cell swelling [40].

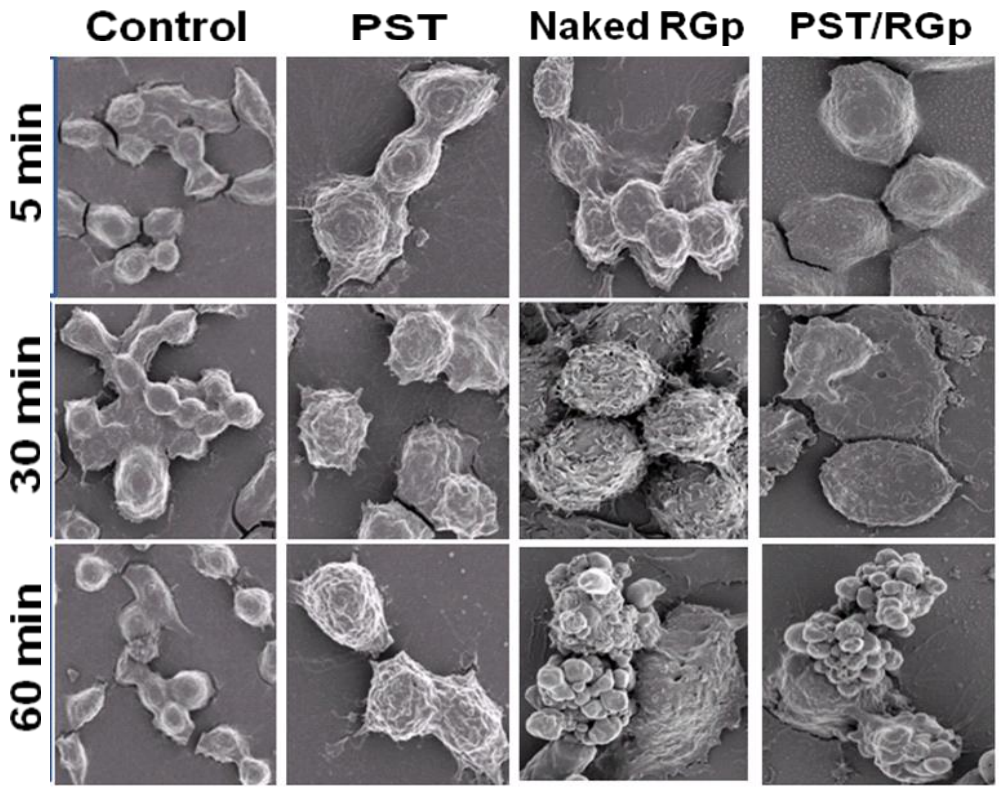


Figure 2-6. Macrophage activation after treatment with PST/RGp complexes at various time periods. Surface morphology of RAW264.7 cells treated with PST, naked RGp, or PST/RGp complexes, evaluated by SEM (magnification 10,000x).

2-3-7-2. Post-uptake Intracellular processing and trafficking of PST/RGp complexes in macrophage

The internalized materials are usually trafficked from acidic endosomes to lysosomes where degradation occurs [41]. Therefore, endosomal escape of the internalized antigens to the cytoplasm is an important step in antigen processing and presentation [41]. It was previously shown that pH-sensitive nanoparticles containing OVA-conjugated polypropylacrylic acid (PPAA-OVA) delivered OVA into the cytosol, leading to a significant improvement of the OVA-specific antibody response [42]. **Figure 2-7A** showed that cells started to uptake PST/RGp complexes after 30 min, presumably by phagocytosis. The complexes were observed in phagosomes after 4 h, and some of them were found rupturing the phagosomal compartment, possibly due to the proton sponge effect of the PEI [17, 19] backbone of PST, which would cause phagosome swelling and eventual rupture with consequent release of the PST/RGp complexes into the cytosol. After 5 h, most of the complexes were observed in the cytoplasm, suggesting that PST efficiently transported RGp to the cytoplasm thereby favoring intracytoplasmic processing [43].

2-3-7-3. PST/RGp complexes selectively stimulated phagocytes and were uptaken by enhanced phagocytosis

To better evaluate the internalization of PST/RGp, the FITC-PST/RGp complexes were prepared and used to treat the cells, which were then analyzed by flow cytometry. As shown in **Figure 2-7B**, macrophages internalized the FITC-PST/RGp complexes in a RGp dose-dependent manner. Blocking phagocytosis with Cyt D, the uptake of FITC-PST/RGp complexes was drastically reduced (**Figure 2-7C**), suggesting that the cellular uptake of the complex was occurred through

a phagocytic process. To further confirm the phagocytosis-mediated uptake, the uptake of PST/FITC-albumin complexes was examined, and likewise found to be significantly reduced by Cyt D (data not shown), suggesting that PST/protein complexes are taken up by macrophages by phagocytosis.

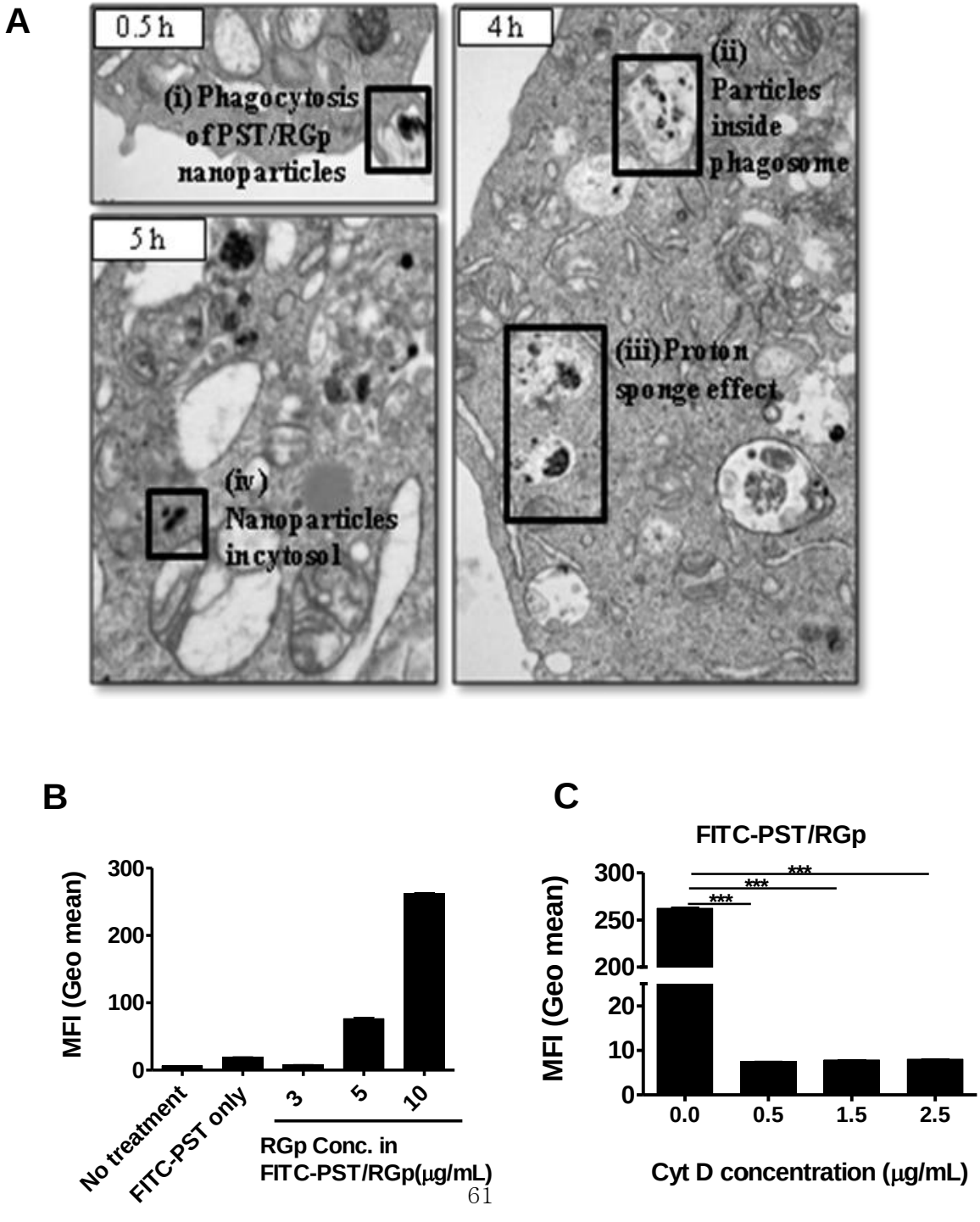


Figure 2-7. Uptake and post-uptake intracellular trafficking of PST/RGp complexes in macrophages. (A) Cellular internalization and intracellular trafficking of PST/RGp complexes (weight ratio 5:1) in macrophages were observed using TEM at different time points (0.5, 4 and 5 h). The boxes indicate the location of the nanoparticulate complexes inside the cells. Magnification 10,000x. (B) Cytofluorimetric determination of cellular uptake of FITC-PST/RGp complexes (weight ratio 5:1) in macrophages at increasing RGp concentrations, in comparison with FITC-PST (50 µg/ mL) and medium as controls. Uptake is expressed as geometric mean fluorescence intensity (MFI) (mean ± SD, n=3). (C) Inhibition of phagocytosis was assessed by flow cytometry on RAW264.7 cells exposed to FITC-PST/RGp complexes (weight ratio 5:1; with RGp at 10 µg/ mL) in the presence of increasing concentrations (0, 0.5, 1.5 and 2.5 µg/mL) of the phagocytosis inhibitor cytochalasin D (Cyt D). Uptake was expressed as Geo MFI value (mean ± SD, n=3). ***P<0.001, by one-way ANOVA.

By comparing uptake of FITC-PST/RGp complexes by macrophages with the uptake by A549 cells, a non-phagocytic lung cell line, it was found that uptake of FITC-PST/RGp complexes was much more potent in the phagocytic cells than in non-phagocytic A549 cells (**Figure 2-8**). While uptake of FITC-PST/RGp complexes by macrophages was drastically reduced by Cyt D, the low uptake by A459 cells was not significantly affected by the inhibitor, confirming that it occurs through a phagocytic process. On the other hand, uptake of FITC-PEI 25kDa/RGp complexes was similar in both RAW264.7 and A549 cells and was not affected by Cyt D, suggesting the uptake by a phagocytosis-independent mechanism. Indeed, PEI 25kDa-mediated internalization has been reported to take place by endocytosis [44].

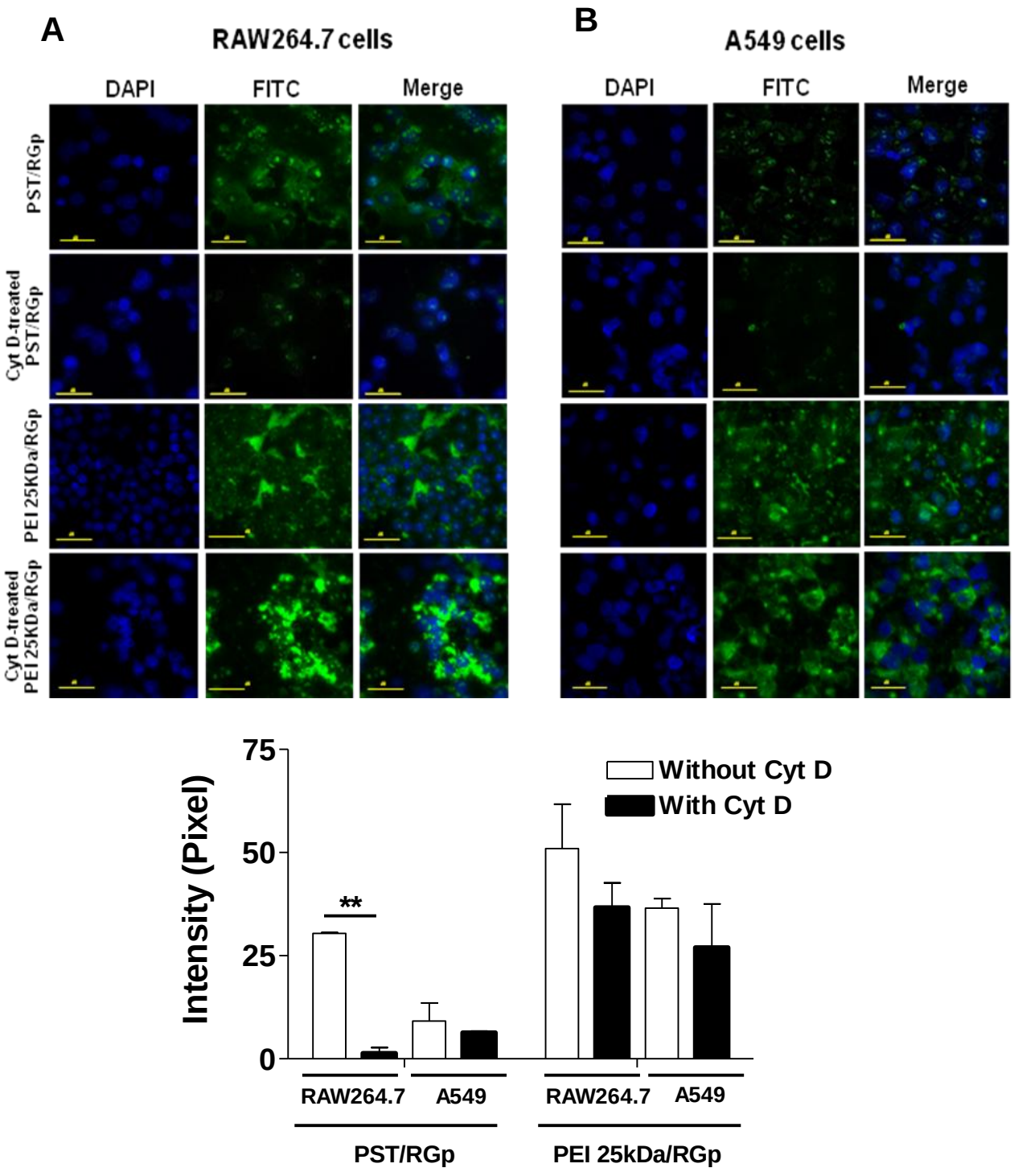


Figure. 2-8. Uptake of FITC-PST/RGp complexes by phagocytic and non-phagocytic cells. RAW264.7 (a phagocytic macrophage cell line) and A549 (a non-phagocytic lung epithelial cell line) cells were pre-treated with 1.5 μg/mL Cyt D for 1 h and then exposed to FITC-PST/RGp

complexes (weight ratio 5:1) for 30 min. After proper washing and fixation, the cells were mounted with DAPI containing fluoroshield solution for nuclear tracking and protecting fluorescence intensity. (A) Cells were observed through image restoration microscopy (IRM) (magnification: 40x). Uptake of FITC-PST/RGp complexes was compared with that of FITC-PEI 25kDa/RGp complexes (weight ratio 5:1) in phagocytic and non-phagocytic cells. At least 1,000 cells were examined for each sample. Bar denotes 40 μm. (B) Quantitative evaluation of fluorescence intensity, measured with ImageJ software. Data are expressed as the mean pixel value ± SD (n=3). $^{**}P<0.01$, by one-way ANOVA.

The preferential uptake of the PST/RGp complexes by phagocytic cells through a phagocytosis mechanism implies a better targeting to antigen-presenting cells with consequent improved induction of adaptive immunity, including antibody response [45], as confirmed by our *in vivo* study. The long-term persistence of the antibody response could also be occurred due to the polysorbitol part of PST, a polysaccharide that might also play a role in the induction of prolonged memory response [46]. The overall mechanism of PST/RGp system for inducing long-term RGp-specific antibody response is schematically represented in **Figure 2-9**.

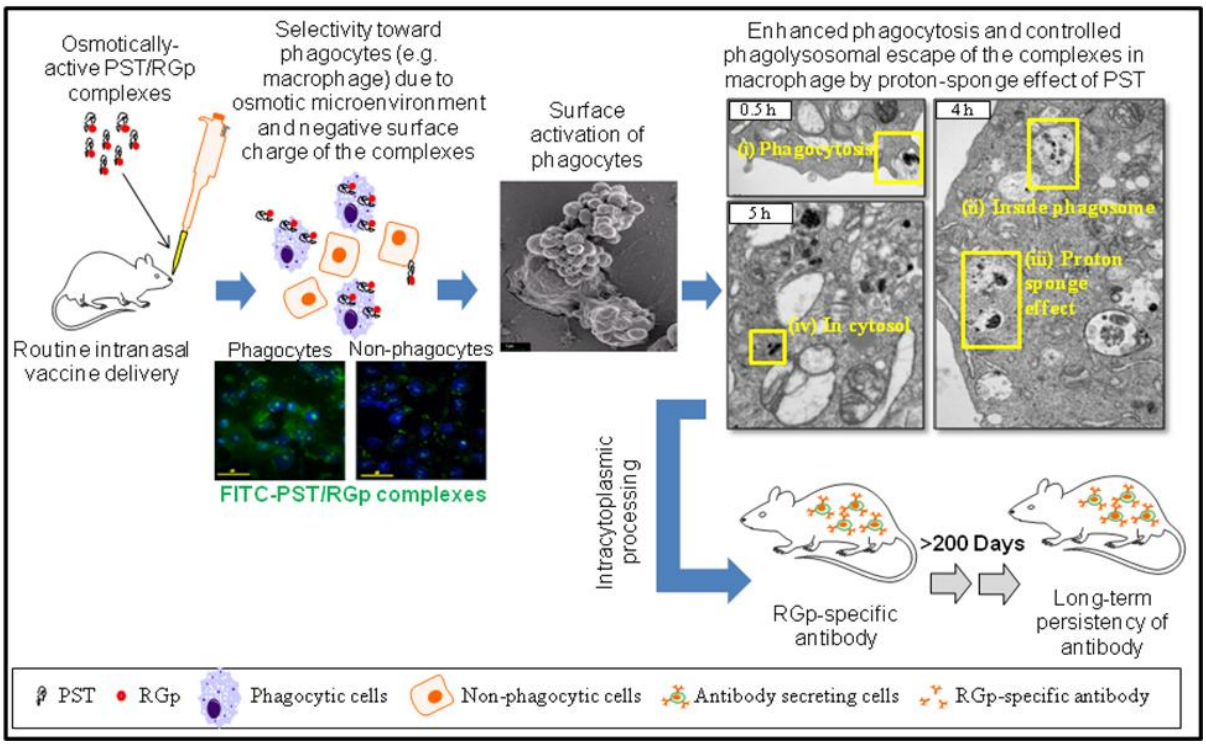


Figure 2-9. Schematic representation on the mechanism of PST/RGp system for inducing a long-term RGp-specific antibody response (figure does not represent the scale of the molecules).

2-4. Summary and Future Prospective

In summary, we have designed and successfully synthesized PST as an improved material for acting as an efficient delivery tool for RGp. PST exhibited stable condensing ability of RGp by simple mixing without any significant damage of RGp functional structure. Remarkably, PST showed excellent safety in both *in vitro* and *in vivo*. RGp delivered with PST showed improved antibody responses and interestingly, the antibody stimulation was persisted for a longer periods of time (>200 days). The mechanism studies showed that the phagocytes were efficiently activated by PST/RGp complexes and phagolysosomal escape was controlled by the buffering capacity of PST allowed successful antigen processing and presentation for induction of specific immune response. The most striking features of PST were its osmotic activity and negative zeta potential which specifically stimulated phagocytes and improved the phagocytic cell-mediated uptake for inducing long-lived antigen-specific antibody production, where the polysaccharide properties of PST also might have synergistic effect. In conclusion, this study indicates PST as a promising adjuvant and functional delivery material for RGp, devoid of side effects and able to induce long-term immunity, thus applicable to develop new vaccine candidates for human use.

2-6. References

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Chapter III

PST Causes CD1d Expression in Phagocytes and B Cells to Induce iNKT Cell Activation

3-1. Introduction

The immune system coordinates with the activity of innate and adaptive immunity to fight against infections. The innate immune system includes antimicrobial compounds, epithelial barriers and various different types of immune cells such as macrophages, dendritic cells, neutrophils and natural killer cells, whereas the major components of adaptive immunity are the B and T-lymphocytes [1]. The main goal of vaccination strategy using adjuvant is to support in the expansion of antigen-specific B and T lymphocytes for protective immunity and, more importantly, memory responses. From the last decade, an unconventional T lymphocyte named natural killer T (NKT) cell, plays essential role as an interface between innate and adaptive immune responses, has been popularly studied [2]. This cell is capable to induce rapid responses, resulting in the activation of antigen-presenting cells and facilitating the development of adaptive immunity. During T cell maturation, most of the auto-reactive cells in the thymus are destroyed to prevent autoimmunity; however some NKT cells express a semi-invariant T cells receptor termed as invariant NKT (iNKT) cells [3]. It was found that iNKT cells (also considered as innate T cells) can recognize glycolipid antigens presented by CD1d and provide help to B-cells after stimulation by agonist compound such as alpha-galactosylceramide (α-GalCer) [4]. Several other compounds including alpha-galactosylceramide (α-GalCer) can activate iNKT cells *in vitro* and *in vivo* [5, 6].

α-GalCer, known to act as an effective adjuvant against infections [7–9] and tumors [5, 10], is a glycolipid ligand presented by the CD1d molecule, locates on most of the antigen presenting cells (APCs), which binds and activates iNKT cells. After stimulation, the key feature of iNKT cells is their rapid stimulation of various surface molecules and secretion of cytokines which activates various cells of innate and adaptive immune system such as dendritic cells, NK cells, T

and B lymphocytes [9, 10]. They also promote B cells responses initiating a rapid and strong germinal center reaction with a robust stimulation of primary immunoglobulin G (IgG) [4]. It is become clear that iNKT cells play crucial role both in innate and adaptive immunity acting as an interface between them for controlling infections and also represent vital role in vaccination strategy [3, 5].

It was also reported that the intranasal administration of α-GalCer increased lung eosinophilia by the action of iNKT cells in OVA-immunized and -challenged mice [11]. The activated iNKT cell can also play an important role in the accumulation of neutrophils in the infection site [12]. The essential role of neutrophil in B cell development has been reported recently and they are also thought to be one of another very useful immune cell to make the bridge between innate and adaptive response [13]. It is worthy to mention that the migration and maturation of these neutrophils is greatly regulated by the recruitment of eosinophilia [14]. Therefore, all these evidences suggest that iNKT cells are very important immune components that control the immune community those are directly responsible of inducing adaptive immune response. In a recent report, interestingly, Cantu et al. described that the activation of iNKT cells depends on the kinetics of antigen binding and suggested that the sugar moiety of α-GalCer is critical in that interaction [15].

In my previous study, it was found that PST acted as a promising delivery system and adjuvant to induce long-lasting antigen-specific antibody responses. However, at this moment it is still unclear which molecules are involved and responsible for this outcome.

Thus, it will be interesting to investigate whether sugar moiety of PST has any effect in the activation of iNKT cells-mediated immune response. Moreover, PST has polysaccharide backbone and previous report showed that polysaccharide antigen can activate B cells [16]. Therefore, it was hypothesized that the structural properties of PST as described above can greatly aid to the stimulation of antigen-specific immune response with long-term persistency possibly regulating by iNKT cells activation.

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3-2. Materials and Methods

3-2-1. Materials

Polysorbitol Transporter (PST) was prepared as reported previously [17]. The EndoFit™ ovalbumin was purchased from InvivoGen (Sorrento Valley Blvd. San Diego, CA, USA). Cy5.5NHS ester and Rhodamine Isothiocyanate (RITC) were purchased from Lumiprobe (33009 Florida, USA) and Sigma-Aldrich (St. Louis. MO 63103, USA), respectively. Alexaflour488 conjugated Ovalbumin was purchased from Life technologies (3050 Spruce St. St. Louis, USA).

3-2-2. Animals

Six week-old female C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained throughout the study in a controlled environment at 24 ± 2°C and 50 ± 10% relative humidity with a 12 h light/dark cycle. All procedures were performed in accordance with rules and regulations of Institutional Animal Care and Use Committee, Seoul National University (IACUC No.: SNU-140326-9).

3-2-3. Physicochemical characterization of PST/OVA complex

Morphology, particle size, surface charge of PST/OVA complex at different ratio was evaluated.

The ability of PST to complex with OVA was determined by transmission electron microscopy (TEM). Briefly, PST/OVA complexes were prepared at various weight ratios by incubating the components at room temperature in distilled water for 30 min, in a total volume of 2 mL with a final protein concentration of 40 µg/ mL. A single drop of complexes was placed on the copper grid and stained with 1% uranyl acetate solution for 10 seconds followed by several washing with DW. The grid was dried for 10 min and the morphology of PST/OVA complexes was observed by TEM (JEM1010, JEOL, Japan). Particle size and surface charge of PST/OVA complexes were measured by dynamic light scattering spectrophotometer with 90° and 20° scattering angles, respectively, at RT.

3-2-4. Detection of CD1d expressing OVA+ APCs, neutrophils and in contrast CD1d receptor (CD1d tetramer) expressing iNKT cells *ex vivo* and *in vivo*.

Ex vivo experiments were designed for preliminary screening. Lung (after perfusion), spleen, liver, CLN, MdLN were isolated from untreated naïve C57/Bl6 mice; single cell suspension was prepared and treated with only media, OVA, PST/OVA and CT/OVA for different time points i.e. 1hr, 3hr, 6hr and 12hr. And then the cells of interest were detected.

In vivo study, C57/Bl6 mice were intranasally administered with PBS, OVA, PST/OVA and CT/OVA and after 3 hours, mice were sacrificed and lungs and MdLNs were isolated. Single cell suspension was prepared.

One set for detecting CD1d expressed macrophage, dendritic cells and neutrophils thus stained with the following antibodies: FITC-labeled anti-mouse CD11c (Clone poly4053, Jackson Immunoresearch); PE-Cy7-labelled anti-mouse CD11b (clone 1D3, Biolegend); PerCP-Cy5.5-labelled anti-mouse CD1d (ebioscience); APC-labeled anti-mouse F4/80 (clone GL-7, BD

Biosciences); BV421-labelled anti-mouse LY6G (clone 281-2, BD Biosciences); and BV650-labelled anti-mouse CD62L (clone 281-2, BD Biosciences). Another set for detecting CD1d expressing B cells and CD1d tetramer expressing iNKT cells thus stained with APC-Cy7-labelled anti-mouse CD19 (clone 1D3, Biolegend); PerCP-Cy5.5-labelled anti-mouse CD1d (Ebioscience); FITC-labeled anti-mouse CD3e (Biolegend), PE-labeled anti-mouse CD1d tetramer (ebioscience).

3-2-5. Detection of CD1d+ OVA+ B cells

And to check whether the CD1d expressing B cells have uptaken OVA, FITC-OVA was administered to detect CD1d expressing OVA+ B cells. Briefly, the cells were counted and stained with premix antibodies for 20 minutes in ice and dark condition followed by PBS washing. Then the cells were resuspended again in PBS and the expression was examined by using flow cytometry and FlowJo software and expressed as MFI (mean fluorescence intensity).

3-2-6. Biodistribution of OVA as naked or complexed with PST *in vivo*

To compare the biodistribution of the antigen, OVA was labeled with Cy5.5 mono-NHS Ester (Sigma Aldrich, The Old Brickyard New Road, Gillingham, United Kingdom). In brief, 5 ml of OVA (5 mg/mL) dissolved in a co-solvent (DMSO:PBS= 1:1) was mixed with 400 µL Cy5.5 NHS ester (2.25 mg) in the same solvent with 8 µL of triethylamine in the dark for 12 h with magnetic agitation at room temperature (RT). The mixture was then dialyzed (using a Spectra/Pro® membrane of 3500 MW cut off) for 2 days in distilled water at 4^{o}C. Finally, the conjugate was lyophilized and stored at -70°C until use. PST/Cy5.5-OVA nanocomplexes were prepared. Then 20 µl of these complexes were intranasally administered to C57BL/6 mice. After

three hours, the mice were sacrificed, organs isolated, and images were captured using imaging system- eXplore Optix (Collip Circle, London, Ontario).

To examine closely on the biodistribution of OVA inside organ, tissue sample was prepared after treating mice in the same way. Alexaflour488 conjugated OVA (Biolegend) were used as naked or in complexation with PST labeled with rhodamine isothiocyanate (RITC-PST). In brief, rhodamine isothiocyanate (RITC) (Sigma-Aldrich) was conjugated to PST (4.3 mg/mL) dissolved in sodium bicarbonate buffer (100 mM at pH 8.0) was mixed with a RITC solution (0.5 mg/mL) in the same buffer. The mixture was stirred at RT for 12 h followed by dialysis (using a Spectra/Pro® membrane of 3500 MW cut off) for 2 days in distilled water at 4°C. Finally, the conjugate was lyophilized and stored at -70°C until use. RITC-PST/Alexa488-OVA complexes (weight ratio 5:1) were mixed and incubated at RT for 30 min before the last immunization. Alexa488-OVA without PST were administered as a control. After three hours, mice were sacrificed, organs collected, and fixed with 4% PFA for 2 h followed by PBS washing for two times and submerged in sucrose solution for overnight. All organs were then inserted into OCT mold and immediately kept in -70°C for storage. Tissues from these organs were plotted on glass slide (Marienfeld, Germany) after cryosection. Then, the slides were mounted with DAPI containing fluoroshield solution. After sealing with cover glasses, appearance of fluorescence was examined using confocal laser scanning microscopy (Carl Zeiss LSM710, Leica Co., Germany).

3-2-7. Statistical analysis

The data are reported as mean ± SD, and statistically significant differences between samples were determined by the one way ANOVA, considering as significant P values <0.05.

3-3. Results and discussion

3-3-1. Physicochemical characterization

3-3-1-1. PST stably complexed OVA with suitable physicochemical properties

The morphology, size and surface charge of polymeric carriers are important parameters for their effectiveness, toxicity, biological fate (cellular uptake, biodistribution), and efficacy. To evaluate whether PST is forming suitable particle size and surface charge with ovalbumin in a similar manner with RGp [17], PST/OVA complexes were prepared (at weight ratios of 0.5, 1 and 5) and observed by TEM. Both TEM and DLS data, it was found that PST stably complexed with OVA at 5:1 ratio, forming uniformly distributed compact particles (**Figure 3-1A**). It was apparent that PST could not form stable complexes with OVA at ratio of 0.5 or 1 which was further confirmed by DLS result **(Figure 3-1B)**.

Mesh-pore size of the mucus layer should be considered for delivering the exogenous agent, which is expected to be diffused there [18]. EM analysis reported the nasal and lung mucus mesh spacing between 20 and 200 nm [19]. Average size of PST/OVA complexes was 171.5 nm shown by size distribution (**Figure 3-1B**), suggesting that the PST/OVA nanocomplexes could be favorable to diffuse through the nasal mucus and, therefore, suitable for intranasal vaccine delivery.

The average surface charge of PST/OVA nanoparticles was -11.8±1.4 mV, suggesting no stealth effect and efficacious phagocytosis of the negatively charged PST/OVA nanoparticles.

3-3-1-2. Secondary structure of OVA was retained in complexation with PST

Next, the structural integrity of the OVA complexed with PST was determined. The secondary structure the OVA after complexing with PST was examined by CD and compared to that of

OVA alone. No significant change in the secondary structure of OVA was found (**Figure 3-1C**). Both showed a decline at 220-230 nm of wavelengths. This structural stability might be mediated by the hydrophilic feature of PST to retain the structure of OVA.

3-3-2. Selectivity of organ and tissues by OVA complexed with PST

To check the selectivity of PST/OVA towards the organ and cell types, *ex vivo* experiment was performed. From *ex vivo* data it was found that the number of CD1d expressing cells was decreased at 6 and 12 hours especially in lymph nodes from PST/OVA treated mice compared to 1 hour and 3 hour treatment [Data not shown]. At 3 h of treatment was considered since it showed highest number of CD1d expressing cells. On the other hand, it has been reported that naked OVA almost fully degraded within 3 h of in vivo administration [20]. Thus it is considered that the time when OVA delivered alone is in denatured status, and PST/OVA treated mice is having higher number of CD1d expressing cells. To have a transparent concept about the hypothesis, *in vivo* experimental design was planned. Therefore, 3 h of treatment is considered to investigate the mechanism of PST/OVA system.

To address the question on the biodistribution of OVA, it was conjugated with Cy5.5 and then complexed with PST. The result showed that the signal was higher in PST/OVA treated mice lung compared to the naked OVA treated one which support the concept of sustained antigen better than naked OVA at the target site.

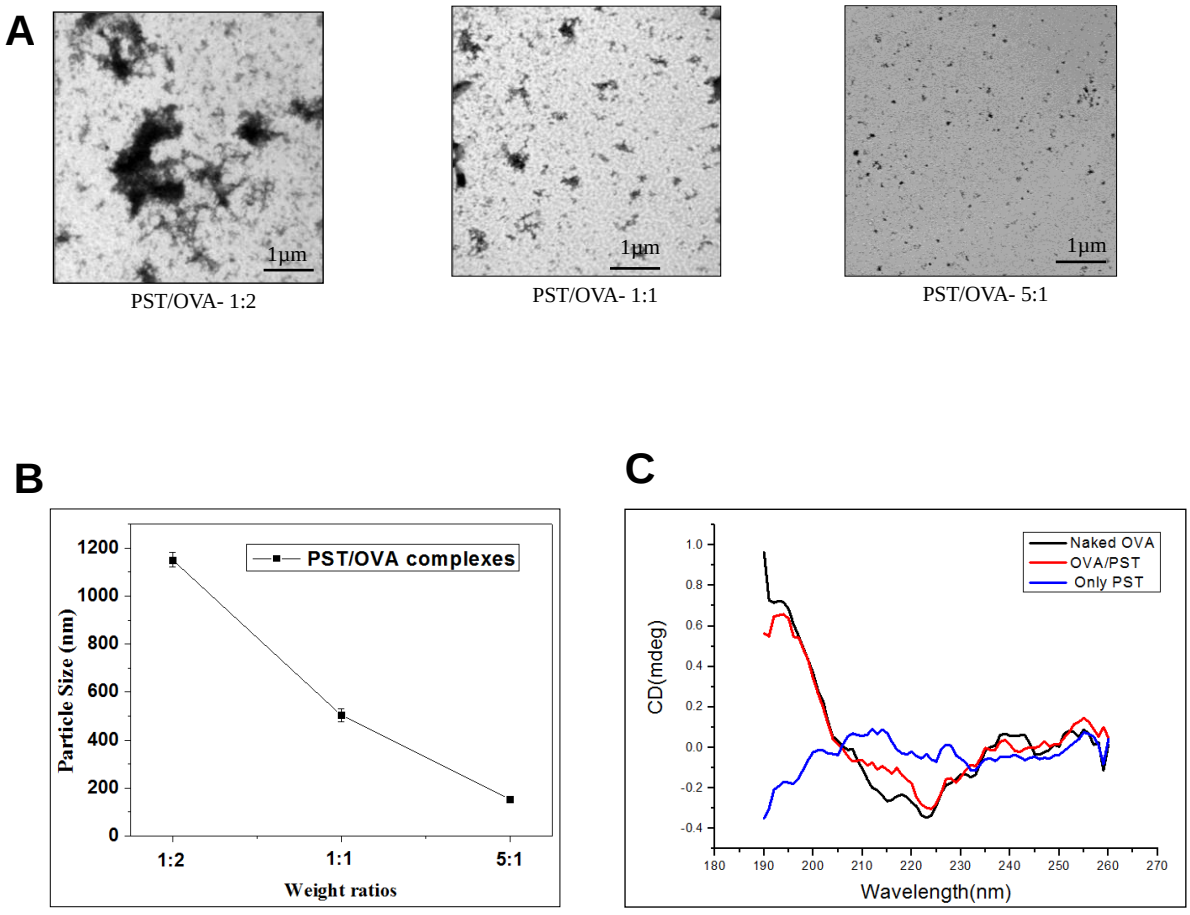


Figure 3-1. Physicochemical and *in vitro* characterization of PST/OVA complexes. (A) PST and OVA were mixed at weight ratio of 1:2, 1:1 and 1:5, and incubated at room temperature for 30 min for complex formation. The images of PST/OVA complexes were captured by TEM (bar denotes 1 μm). (B) Size distribution of PST/OVA complexes at different weight ratio of 1:2, 1:1 and 5:1 was measured using DLS. (C) The structure of complexed OVA was examined by CD spectroscopy and compared with naked OVA. ***P<0.001, by one-way ANOVA.

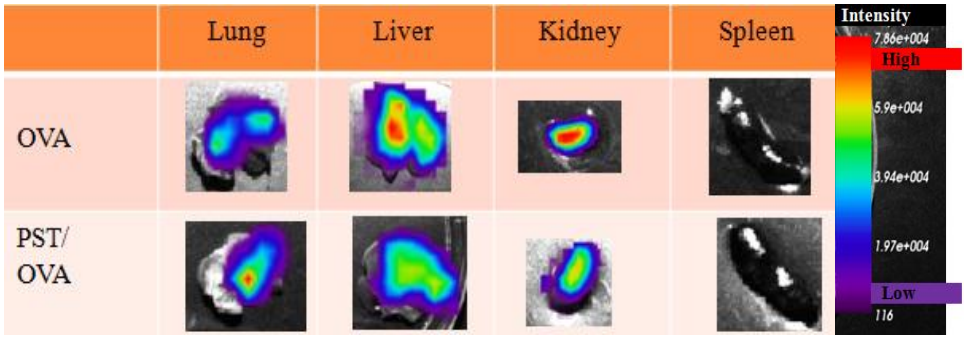
As expected, high amount of OVA was found in the liver and kidney from naked OVA treated mouse suggesting fast degradation and catabolism **(Figure 3-2 A)**. It was noting that the other major immune organs like spleen **(Figure 3-2 A (left panel-last column)** and lymph nodes (data not shown), I could hardly find some signal of Cy5.5 in the whole organ might be either due to the very little amount of immune cells in comparison to the organ size or might be due to technical limitation. To overcome this issue, tissue sections of nasal associated lymphoid tissues (NALT), lung, mediastinal lymph node (MdLN), cervical lymph node (CLN) and spleen from treated mice were prepared. For this purpose, Alexa-OVA without or with RITC-PST were intranasal administered to the mice and sacrificed after 3 h for organ collection.

OVA was found at the local site of administration in mice treated with PST/OVA (**Figure 3-2B i)** including NALT, lung and MdLN, whereas it was not found in mouse treated with naked OVA indicating sustained release of OVA is mediated by PST.

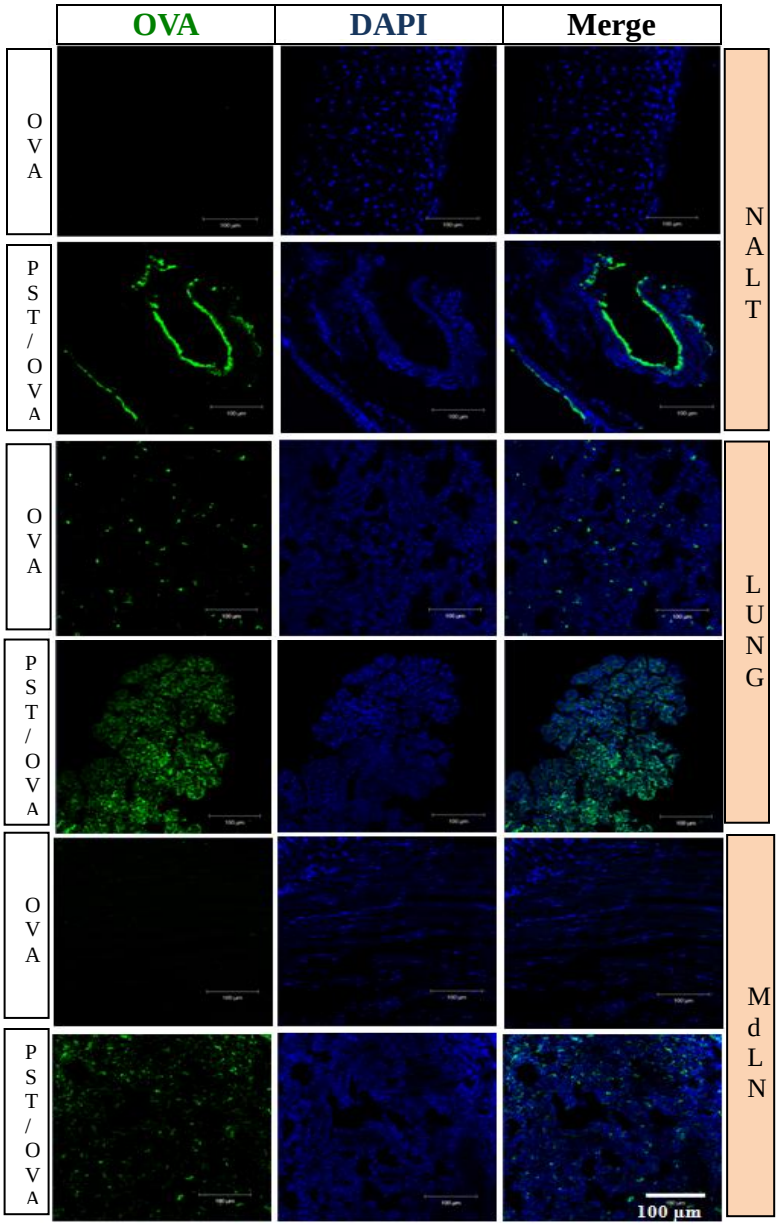
Importantly, in NALT, PST/OVA was found mostly at the extracellular region rather than inside the cell, indicating that these complex were leaving behind this nasal site to proceed for the next target site i.e. lung and secondary lymphoid organ near lung. In CLN and spleen, naked OVA showed some Alexa signal of OVA which might be in the process of degradation since it is reported that at 3 hour of administration of naked OVA, the amount of degraded OVA is found in the maximum level [20].

In order to examine the role of PST on the protection of OVA from degradation, RITC-PST and Alexa-488-OVA complexes were further analyzed for the colocalization. The result showed that PST highly co-localized with OVA in all organs and tissues including CLN and spleen (**Figure 3-2B ii**) suggesting better stability of PST/OVA.

A


Lung
Liver
Kidney
Spleen
OVA
PST/
OVA
Intensity
7.86e+004
High
5.9e+004
3.94e+004
1.97e+004
Low
116

B (i)


OVA
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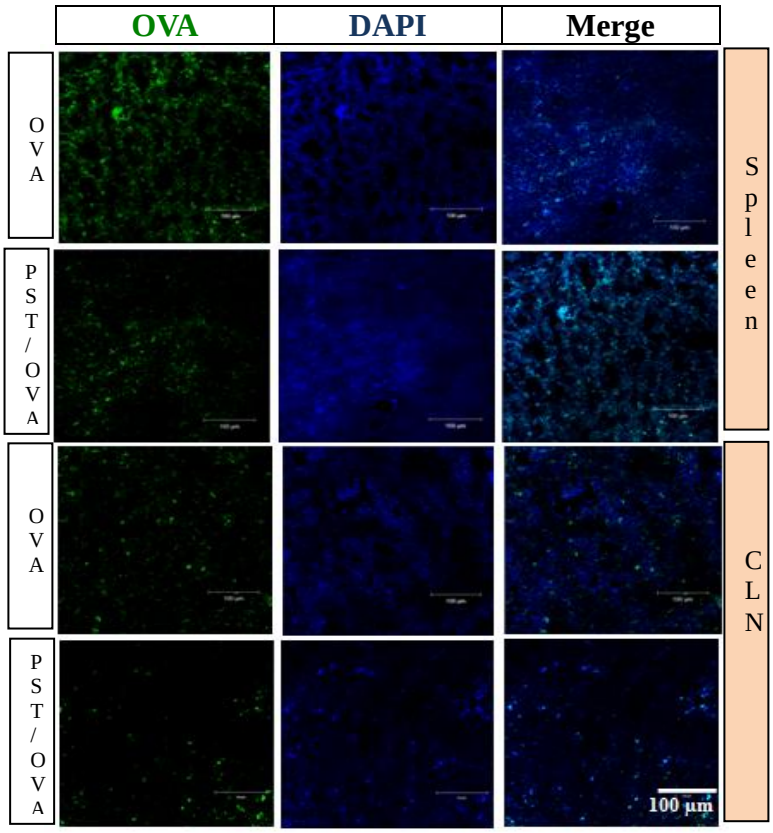

(ii)

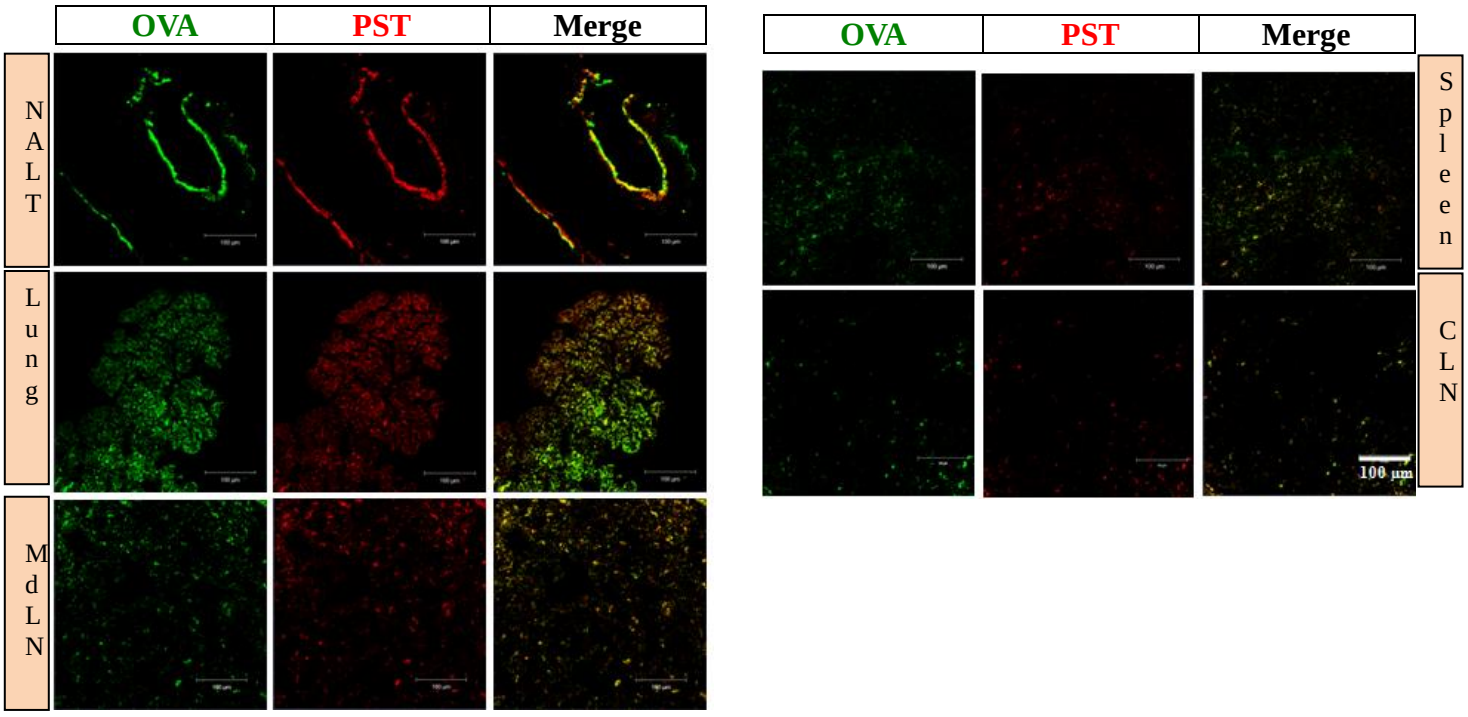

Figure 3-2. Biodistribution of the OVA complexed with PST in Mice were immunized with OVA

with or without PST. A) Lung, liver, kidney and spleen, and B) tissue sections of lung, NALT, MdLN, CLN and spleen were studied. i) Level of Alexa 488-OVA in tissue sections from naked OVA and PST/OVA immunized mice ii) Co-localization of Alexa 488-OVA and RITC labeled PST in tissue sections of lung, NALT, MdLN, CLN and spleen from OVA/PST immunized mice. Scale bar denotes 100 μm.

3-3-3. Increased number of CD1d expressing cells involved in bridging innate cells to B cell development for PST/OVA system

Then I was curious to see what types of cells have uptaken OVA complexed with PST in lung and MdLN. To address this issue, single cells from lung and MdLN of Alex-OVA/PST treated mice were analyzed by using flow cytometry [Data not shown]. In the lung, about 57% cells were OVA+CD19+ i.e. OVA-positive B cells and the rest of the cells were OVA+ CD11c+CD11b+ i.e. two major professional phagocytes (macrophage and dendritic cell) were OVA-positive. This result indicates that the PST/OVA system is strikingly interacting with APCs. When cells from MdLN were analyzed and surprisingly it was found that about 87% of OVA+ cells were B cells. This result suggests that PST/OVA system maintained a high percentage of B cell populations which might be interacted rapidly with the polysaccharide backbone of PST at MdLN which is a major site to bridge innate and adaptive response with a long-term immunity.

However, the signaling mechanism which was required to generate these long-lived plasma cells is still a matter of question. Based on the previous results other published reports, I can come up with conclusion that i) PST itself is not behaving as antigen but protecting OVA to retain its antigenic property; ii) PST/OVA not only showed antibody production but also the antibody

persisted for long-periods of time; iii) at early time point (3 h) after immunization PST was found colocalized with OVA as a complex system; iv) Interestingly, PST/OVA showed distinguishable features from naked OVA at early time (3 h) even at the cellular level of draining LNs. Thus, there might be additional mechanism which potentiated PST-(half life is lesser than 15 days; from previous part) mediated antigen delivery at the early time point that functionally might be linked with the length of antibody persistency.

One of the previous report showed that the cognate recognition between B cell and iNKT cell via CD1d played important role on B cell development for inducing long-lived plasma cells [16, 21]. Moreover, CD1d is not only expressed in B-cells but also expressed in macrophage, dendritic cells, neutrophils and some epithelial cells. On the other hand, iNKT cells interacts with these cells and takes part in modulating immune system via its T cell receptor (TCR) by interaction with CD1d ligands of those immune cells [22, 23]. I believed and hypothesized that this interaction might also be involved in PST mediated mechanism, in which PST is seemingly hooking the immune system due to its polysaccharide backbone where CD1d expressing cells might contribute greatly. Unlike other reported antigenic polysaccharide, PST is synthetic polysaccharide which is non-immunogenic on its own and biodegradable delivery tool, however provides all possible favor to the antigenic vaccine to induce antibody response and moreover, triggering plasma B-cells to reach at their long-lived niches at a specific site for clonal expansion to provide long-lasting immune response which is very interesting. .

To address whether PST is inducing this interaction via CD1d expressing cells, I immunized mice with PBS, OVA, PST/OVA and CT/OVA as similar manner mentioned earlier. Mice were sacrificed after 3 h and lung and MdLNs were isolated. Single cell suspensions were prepared and stained with FACS antibody to detect B cells, macrophage, dendritic cells, and neutrophils

with CD1d expression and iNKT cells with Cd1d tetramer (receptor for CD1d). **Figure 3-3A i** shows that all different types of CD1d positive phagocytic and antigen presenting cells are presented in a significantly higher number in lung for PST/OVA-treated mice compared to other groups. However, in correspondence with the increased CD1d positive cells in lung, the $CD1d^{tet}$ expressing iNKT cells was not changed unexpectedly; indicating that there was no activation of iNKT cells in lung. In MdLN, all the aforementioned CD1d positive cell types was also found in higher number for PST/OVA-treated mice (**Figure 3-3A i**), in fact in a very high amount compared to others. But unlike lung tissue, it was very interesting to observe that the $CD1d^{tet}$ expressing iNKT cells in MdLN showed a robust increase in number for the PST/OVA-treated mice, strongly suggests that iNKT-mediated OVA-specific B cell development requires germinal center for PST/antigen system where CD1d plays a very crucial role for induction of prolonged immune response.

It was reported that, when lipid antigen such as αGalCer was co-administered with (but not directly conjugated to) a protein antigen (so that lipid recognition by iNKT cells can occur separately from antigen recognition by B cells) iNKT cells can still provide efficient B cell help [24, 25]. Indeed, activated iNKT cells can provide B cell help [26], and this does not require germinal center localization [27]. In our study, PST/OVA did not activate iNKT cells in lung in the absence of germinal center, confirming that e PST supports (co localization data shows their co-existence) the protein antigen, OVA who needs germinal center to undergo somatic hyper mutation and affinity maturation, resulting in generation of long-lived plasma cell or memory B cells. Furthermore, according to the previous report, only αGalCer was recognized by iNKT cells resulted in activation without germinal center. In my study, since antibody response to protein antigen requires participation of both T cells and B cell of germinal center within

lymphoid follicles, as naked antigen OVA could not activate iNKT cells in lung. And it might be possible that those CD1d got bound to NK cells. Interestingly, PST (presumably with its polysaccharide backbone) has done the trick to activate iNKT cell similarly as αGalCer; Therefore OVA complexed with PST modulate iNKT cells and CD1d expression as bridge to translate innate response to long-lasting B cell response. Patrick et al. reported how iNKT cell based activation can do function; the important information is summarized in **Table 3-1**. According to the promising results of the present study a schematic representation of the molecular mechanism of PST on how it works for inducing long-lasting immunity is shown in **Figure 3-5**.

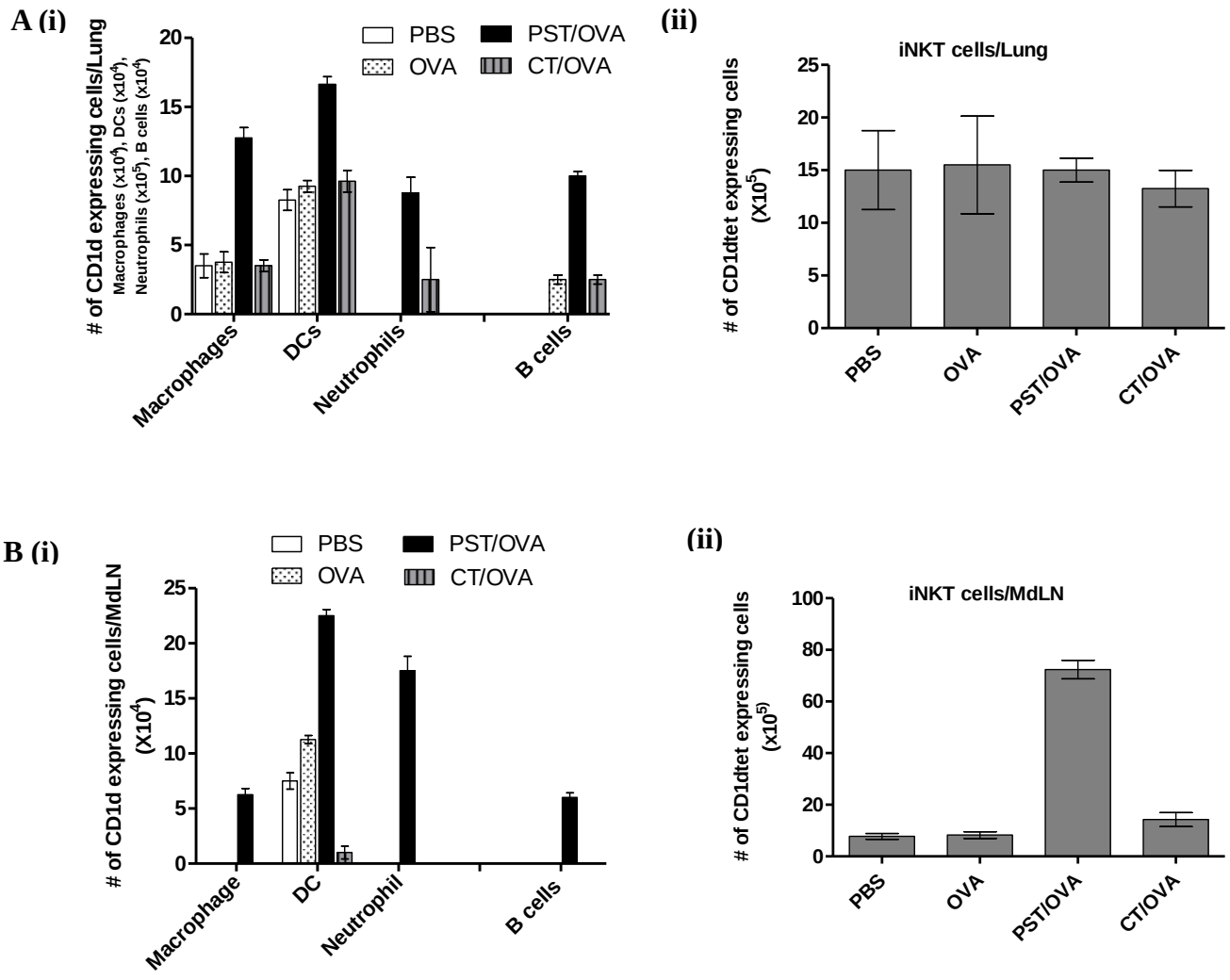

Figure 3-3. Contribution of CD1d positive phagocytic and antigen presenting cells to activate iNKT cells in MdLN that served as a site of bridging innate to adaptive immune system. Mice were immunized with PBS, OVA, PST/OVA and CT/OVA as similar manner mentioned earlier. Mice were sacrificed after 3 h followed by isolation of lung and MdLNs. (A) Detection of (i) CD1d expressing B cells, macrophages, dendritic cells and neutrophils and (ii) $CD1d^{tet}$ [receptor for CD1d] expressing iNKT cells in lung. (B) Detection of (i) CD1d expressing in B cells, macrophages, dendritic cells and neutrophils and (ii) $CD1d^{tet}$ [receptor for CD1d] expressing iNKT cells in MdLN.

Table 3-1. Mechanism of iNKT cells activation through interaction of phagocytes and (or) APCs

Interaction (CD1d restricted)	**Cytokines involved**	**Mechanisms for inducing long-lasting immunity**
Dendritic Cells-iNKT cell Or Macrophage - iNKT cell	IL-4, GM-CSF, IFN, TNF IL-4, IL-13, GM-CSF, IFN, TNF	Express CD1d as APC and interacts with iNKT cells which leads to iNKT cell activation and reciprocal APC activation or modulation. The major iNKT cell-derived cytokines that modulate the functions of the iNKT cell cognate partner during immune activation.
B cell - iNKT cell	IL-4, IL-10, IL-13, IL-21, IFN	iNKT cells interacts directly with B cells for inducing long-term antibody response
Neutrophil- iNKT cell	IL-17A, GM-CSF, IFN, CXCL2	iNKT cells induce neutrophils migration at infection site. These neutrophils help B cells to function

3-3-4. CD1d expressing B cells are OVA+ in PST/OVA treated mice: polysaccharide backbone of PST is a key factor

As hypothesized earlier another possibility is that the nature of polysaccharide backbone of PST could offer enhancement of antigenic interaction with the immune cells, especially B cells of

draining lymph node and successful delivery of OVA showed long-term antibody response. Earlier, it is found that specifically PST is showing this effect, not its individual components such as SDA or BPEI or an unreacted mixture of SDA plus BPEI. Therefore, it is understandable that the chemistry of PST might make the differences to show a stable antibody response for longer periods of time. Thus I was highly interested to check the involvement of any molecular compound (such as protein) on the interaction between the polysaccharide backbones of PST with APCs, which might be resulted in long-term persistency of antibody. To address that, I have checked whether the CD1d expressed cells are also OVA+ or not. In **Figure 3-4 (A)**, only PST/OVA treated group showed a significant number of OVA+ CD1d expressing B cells. This interesting result was due to the polysaccharide backbone of PST or not is still a question. To solve that, in **Figure 3-5 (B),** another polymeric adjuvant PEI which does not have polysaccharide backbone was compared with PST. It is mentionable that, PEI is also one of the components of PST, in which the other component SDA is responsible for polysaccharide backbone of PST. Interestingly the result showed that in PSI/OVA treated mice the CD1d expressing cells are not OVA+ i.e. they did not uptake antigen. To check this CD1d expressing B cell-antigen binding results in long term antibody response or not, mice were immunized with the same reactants (SDA and BPEI) individually or mixture and OVA-specific IgG was measured at day 7 and day 90 after last immunization. Interestingly, **Figure 3-4 (C)** showed that the BPEI itself and the mixture of SDA plus BPEI showed a minimal level of OVA-specific antibody at the very early time point and did not last for longer period of time.

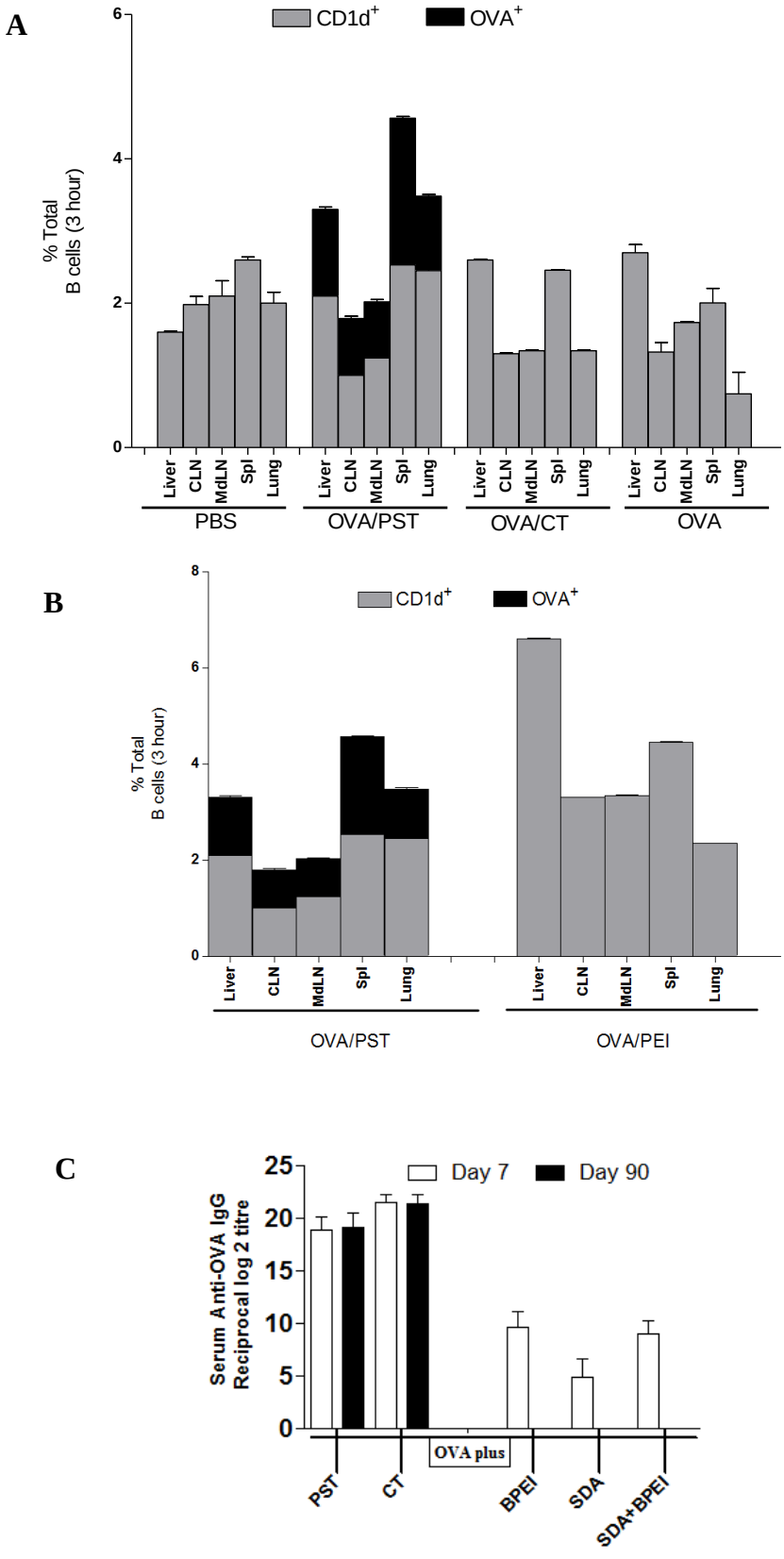
A
CD1d+
OVA+
% Total
B cells (3 hour)
Liver
CLN
MdLN
Spl
Lung
PBS
OVA/PST
OVA/CT
OVA
B
CD1d+
OVA+
% Total
B cells (3 hour)
OVA/PST
OVA/PEI
C
Day 7
Day 90
Serum Anti-OVA IgG
Reciprocal log 2 titre
OVA plus
PST
CT
BPEI
SDA
SDA+BPEI

Figure 3-4. CD1d positive B cells are also OVA positive in PST/OVA treated mice. Mice were intranasally administered with PBS, OVA, PST/OVA, CT/OVA and also OVA/PEI. Mice were sacrificed after 3 h followed by isolation of lung, liver, CLN, spleen and MdLNs. (A) Detection of OVA+ CD1d expressing B cells and (B) A comparison between PST/OVA with PEI/OVA (a non-polysaccharide polymeric adjuvant) in terms of OVA+ CD1d expressing cells. (C) Effect of polysaccharide backbone of PST on serum OVA-specific IgG persistency. Long term OVA-specific IgG in mice immunized with the components of PST i.e. BPEI or SDA or SDA+BPEI (no chemical reaction, simple mixing) and compared with PST/OVA and CT/OVA.

3-4. Summary and future prospective

In summary, this study showed the mechanism of a well-designed and successfully synthesized PST which its excellence with dual role as an efficient delivery tool and effective adjuvant for antigens (such as OVA) by showing additional mechanism of activating iNKT cells. The chemistry of PST showed their involvement in long-term antibody response, which was triggered at the beginning. In this particular study, I have encountered the functional and mechanistic difference of naked OVA and PST/OVA at very early time which are thought to be involved by synergistic effects of many different kind of cells of innate immune system, few of the cells from adaptive immune system and additionally some cells who has the ability to translate from innate to adaptive situation, thus serving as a bridge between them. It was found that PST/OVA targeted all phagocytes and mostly B cells especially in MdLN which also provided platform for the interconnection with iNKT cell activation in the presence of germinal center. Interestingly, this response was happened for the higher CD1d expression by macrophage, dendritic cells, B cells and neutrophils. Taken together, overall results showed that PST-mediated OVA delivery induced

CD1d expressing phagocytes (macrophage, dendritic cells and neutrophils) and B cells which resulted in robust long-lasting immune response via iNKT cell activation. The possible mechanism of inducing CD1d positive cells could be promising topic for future study and more the role of eosinophils can also be an interesting topic for further study; however, at this moment I can say that it might be due to the unique chemical properties of PST, which has a structure similar to sugar molecule (sorbitol chain). Bruno et al. already patented that these type of sugar alcohols (i.e. sorbitol, xylitol, mannitol) can bind directly to the graft of CD1d because the sugar moiety of the different lipid/polysaccharide antigen also usually follow this mechanism [33]. In this study, sorbitol chain forming polysorbitol serves as a carrier for the vaccine and plays a crucial dual role as an effective delivery system as well as a potent adjuvant to induce long-pasting immunity.

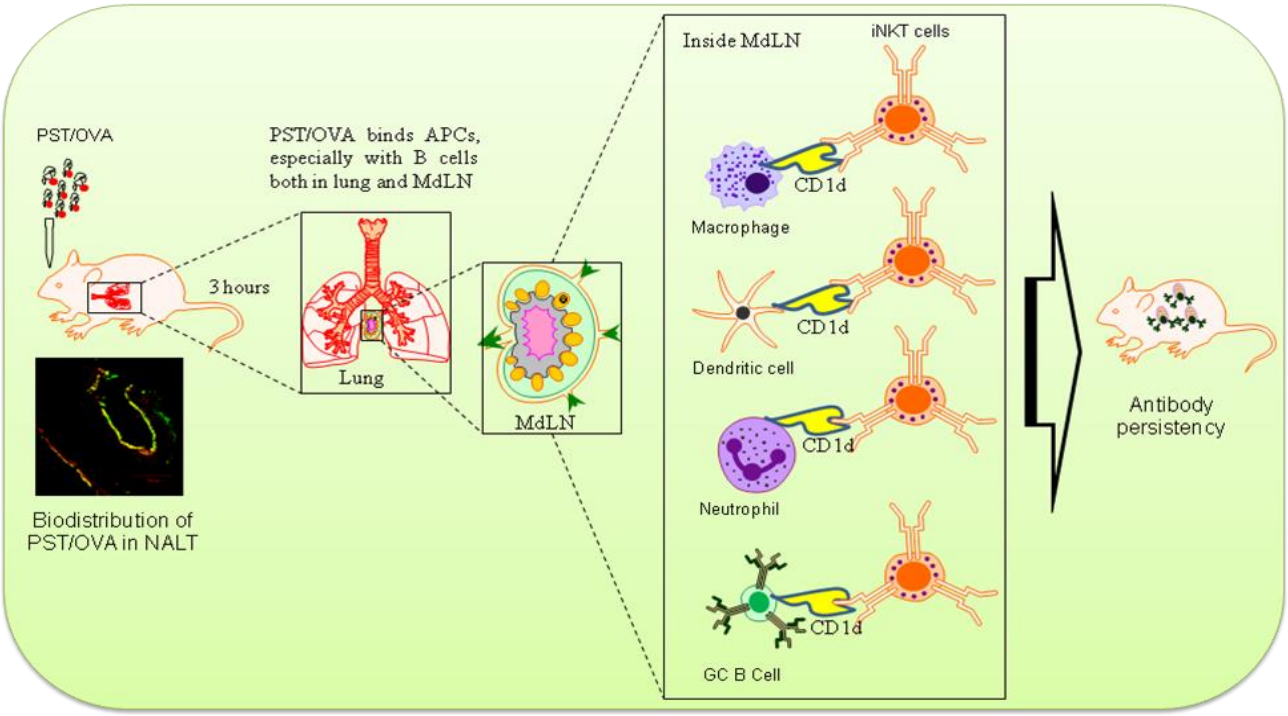

Figure 3-5. A schematic diagram of PST mediated unique action in addition to the conventional mechanism of antibody response.

3-5. References

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Chapter IV

PST Facilitates the Maintenance of Germinal Centre B Cells for Long-Lasting Antigen-Specific Antibody Response in Draining Lymph Node

4-1. Introduction

Adjuvants are the key component of vaccines in order to enhance the antigen-specific immune activity. Development of vaccine candidate with novel adjuvant formulation is advancing rapidly and offering efficacy to fulfill previously unsolved needs; however, most of the new vaccine formulations show inefficacy due to the lack of stability, manufacturability, effectiveness and inappropriate immune responses [1]. Moreover, non-specific immune activity against adjuvants and/or delivery system could trigger immune complication and interfere with bona fide vaccine efficacy. Therefore, the inclusion of adjuvant and delivery system in a vaccine formulation is essentially required and thus, proper therapeutic regimen should be justified [1]. The modern biotechnological advances enable to develop rational design of such vaccine adjuvants (and delivery systems) with improved therapeutic efficacy and safety profile. However, there are still many hurdles to overcome, which are not only limited to (i) simplicity, (ii) tolerability, (iii) inexpensive, (iv) and safety record like alum, but also be beneficiary by (v) antigen dose-sparing, (vi) reduction in boost immunization, vii) longer protection with prolonged immune response, and (viii) better efficacy toward immunocompromised individuals including elderly and neonate [2]. During last decade, extensive research works have been performed aiming to develop practically relevant novel adjuvants and vaccine delivery systems [2, 3]. Especially, the activation of innate immune system initiation, the identification of discrete cell types and immune activation sites which are involved in early phase innate immune activation as well as in the amplifications of immune response have accelerated the advancement in vaccine development research [3]; however, the precise mechanism on how they regulate all these is not well understood.

As described earlier, one of the hallmarks of effective vaccine is to show a long lasting antigen-specific antibody response [4].Designing an adjuvant (as well as delivery system) for efficient immune response, especially for long-term period is challenging [5]. In case of long-term antibody

response, adjuvant facilitates the maintenance of antigen-specific B cells in the secondary lymphoid organs including bone marrow and germinal center (GC) [5, 6]. GC B cells persisted in such a way that they remain functionally active to become plasma B cells to produce secretory antibodies upon exposure to the same antigen.

Biodegradable polymeric nanocarriers are getting into flash light to be applying in adjuvant formulation because of its wide range of tunable features which allow the modification and screening for the best choice based on the size, stability, surface charge and hydrophilicity, hydrophobicity, and the integration of antigenic molecules to maximize immunostimulatory activity of the vaccine [7]. However, 'immunogenicity' of these polymeric materials is rarely addressed. It was reported that poly(carboxybetaine) did not produce antibody against itself and avoided immune system by mimicking cell surface protein structure, which indicates that polymeric materials can be modified for the expected features [2]. In the previous study, I found that PST is safe as vaccine delivery tool, and as adjuvant, is able to stimulate phagocytic cells to provoke prolonged antigen-specific antibody response *in vivo* [5]; however the in-depth mechanism on how PST provided a long-lasting antibody response is yet to be elucidated. Since the immunogenicity against PST itself was not determined, I have tried to address the question on whether PST causes polymer-specific immune response. It, therefore, will be interesting to see how PST supports the vaccine formulation to stimulate immune system and capitalize immuno-stimulatory sites (such as draining lymph nodes) for providing long-lasting immune activity.

4-2. Materials and Methods

4-2-1. Materials

Branched LMW polyethylenimine (PEI; 600 Da), 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl

tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), D-sorbitol, and cytochalasin D (Cyt D) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorbitol diacrylate (SDA) was purchased from Monomer-Polymer & Dajac Labs, Inc. (Trevose, PA, USA). PST was prepared as previously reported [5]. The EndoFit™ ovalbumin was purchased from InvivoGen (Sorrento Valley Blvd. San Diego, CA, USA).

4-2-2. Cell lines

The mouse macrophage cell line, RAW264.7 was obtained from American Type Culture Collection (Manassas, Virginia, USA), and maintained in Dulbecco's Modified Egles's Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2. The cells cultured until about 80 % confluent were sub-cultured every 2-3 days. Unless otherwise stated, the cells were plated at 1 x 10^5/mL/well of 24-well culture plates and grown until about 80% confluent for the experiment.

4-2-3. Animals

Six week-old female C57BL/6 mice were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained throughout the study in a controlled environment: 24 ± 2°C, 50 ± 10% relative humidity and a 12 h light/dark cycle. All procedures were performed in accordance with rules and regulations of Institute of Laboratory Animal Resources, Seoul National University (IACUC No.: SNU-120308-4).

4-2-4. Immunogenicity of PST

4-2-4-1. Biodistribution of PST *in vivo*

To address the fate of PST after nasal delivery, biodistribution was examined by labeling PST with Cy5.5 mono NHS Ester (Sigma Aldrich, The Old Brickyard New Road, Gillingham, United Kingdom). In brief, PST (5 mg/mL) dissolved in a co-solvent (DMSO: DW= 1:1) was mixed with a Cy5.5 NHS ester (2.25 mg) in 400µL of the same solvent with 8µL of triethyl amine in the dark for 12h with magnetic agitation at room temperature (RT). The mixture was then dialyzed (using a Spectra/Pro® membrane of 3500 MW cut off) against distilled water in a dark room at 4^{o}C for 2 days. Finally, the conjugate was lyophilized and stored at -70°C until use. Cy5.5-PST/OVA nanocomplexes were prepared at 5:1 ratio by simple mixing and incubation at RT for 30 min. Then these complexes were intranasally administered to C57BL/6 mice. Prior to imaging, hair was removed from the mice at the region of interest and anesthetized. Images were captured at day 1, 3, 7, 10 and 15 by imaging system; eXplore Optix (Collip Circle, London, Ontario).

4-2-4-2. Cytokines test by ELISA

C57BL/6 mice were administered intranasally with PST (100 and 250 µg/mouse in PBS) or cholera toxin (CT; 2 µg/mL in PBS; intranasally) in a volume of 20 µL as a control. After 24 h, blood samples were collected retro-orbitally and serum was isolated after centrifuging blood at 8000 rpm, 10 min at 4℃. The concentration of TNF-α and IL-12p40 in the serum was determined with commercial ELISA kits (R&D Systems, Minneapolis, USA) following manufacturer’s instruction.

4-2-4-3. Antigen-specific antibody response

C57BL/6 mice (n=6) were immunized intranasally with 20 µg of PST alone or PST/OVA complexes (weight ratio 5:1) suspended in PBS. Positive control mice received 20 µg of OVA mixed with 2 µg of CT. Immunization was performed three times at two weeks interval. Blood samples were collected

retro-orbitally to determine antigen-specific antibody levels in serum on days -28, -14, 0 (3^{rd} or last immunization), 60 and 90.

To measure the presence of antigen-specific antibodies in serum, ELISA plates were coated with PST (10 µg/mL diluted in PBS, 100 µL/well incubated overnight at 4°C) or OVA (5 µg/ mL dilution in PBS), respectively. The wells were blocked with 1% BSA in PBS, washed, and then incubated with serial dilutions of sera in blocking buffer (200 µL/well) for 1 h at 37°C. After washing, antigen-bound antibodies were detected with goat anti-mouse antibodies conjugated with HRP (Southern Biotech, Birmingham, Alabama, USA) followed by 3, 3´, 5, 5´- tetramethylbenzidine dihydrochloride (TMB), as HRP substrate (Sigma Aldrich). After 20 min, the reaction was stopped using 1N H_2SO_4 and the reacted substrate was measured spectrophotometrically at 450 nm using VERSAmax tunable microplate reader (Sunnyvale, USA). Antibody concentrations were determined after normalizing the readings with the plate background using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

4-2-6. Detection of OVA-specific plasma and germinal center B cells

Mice were immunized as aforementioned. Then they were sacrificed on day 7, 60 and 90 after the last immunization and lung (after perfusion), spleen, bone marrow, mediastinal lymph node and cervical lymph node were collected. Lungs were chopped into small pieces and treated with collagenase-DNAse media at 37°c for 3 to 5 minutes. Then, single cell suspension was prepared by gentle crushing of organs through a 70 µM pore filter (BD Falcon) and pooled, followed by ACK lysis buffer (Life technologies, USA) treatment. The cells were stained with proper combination of following antibodies: PE-Cy7-labelled anti-mouse IgG (Clone:poly4053, Jackson Immunoresearch); APC-Cy7-labelled anti-mouse CD19 (clone 1D3; Biolegend); V450-labelled anti-mouse IgD

(Ebioscience); 7AAD for live/dead cell discrimination (Invitrogen); PE-labeled anti-mouse Ly77/GL7 (clone GL-7; BD Biosciences); V605-labelled anti-mouse CD138 (clone 281-2; BD Biosciences); APC-labeled anti-mouse TCR-β (clone H57-597; Biolegend); APC-labeled anti-mouse CD11b (clone M1/70, BD Biosciences) and Alexa488-labelled OVA (Invitrogen). Briefly, the cells (~10^7/ml) were stained with premix antibodies (mentioned above) for 20 minutes in ice and dark condition followed by PBS washing. The degree of expression was detected using flow cytometric analysis, FACS Canto II (BD Biosciences, San Jose, CA, USA). All cytometric data were expressed as absolute number and analyzed by using FlowJo software (New Jersey, USA). Following the gating, T cells and $CD11b^+$ myeloid cells were excluded, and the $CD19^+$ cells were selected and further gated on IgG+IgD- isotype switched cells. OVA-specific IgG cells were further identified as Alexa-488 labeled OVA+ cells, which were analyzed for the expression of CD138+ and GL-7, to distinguish plasma cell versus germinal center cells. The gating strategy: T cells and $CD11b^+$ myeloid cells were excluded, and the $CD19^+$ cells were selected and further gated on IgG+IgD- isotype switched cells. OVA-specific IgG cells were further identified as Alexa-488 labeled OVA+ cells, which were analyzed for the expression of CD138+ and GL-7, to distinguish plasma cell versus germinal center cells.

To trace OVA-specific cells at 90 days post last immunization, various organs were collected from the mice. The bone marrow was treated with fixative solution (Sodium Phosphate added 40% formalin) for 10 days, then decalcifying solution (10% EDTA solution) for 7 days with both solutions changed every day. Except bone marrow, all other organs were the primarily fixed with 4% PFA for 2 h followed by washing with PBS for two times and soaked down in sucrose solution for overnight. All organs were then inserted into OCT mold (Sakura, Flemingweg, Netherlands) and immediately kept at -70°C for storage. Then, tissues were cryosectioned and plotted in glass slide bar. Then the slides were stained with Alexa488-labelled OVA and mounted with DAPI containing fluoroshield solution.

After sealing with cover glass (Marienfeld, Germany), slides were observed using confocal laser scanning microscopy (Carl Zeiss LSM710, Leica Co., Germany)..

4-2-7. Restimulation of germinal center B cells and plasma B cells with OVA

To check whether plasma cells are originated from germinal center B cells upon antigen restimulation, single cell suspensions were prepared from the organs of immunized mice in a similar procedure as mentioned in **4-2-6 section**. The cells (10^5cells/well) were seeded in a 96-well plate and treated with or without OVA (20 μg/mL) in a complete culture medium at 37°C in humidified incubator with 5% CO_2 for five days. On the third day, 200 μL of media containing OVA or media only were added to each well. On day five, the supernatants were collected and OVA specific IgG titers were measured by ELISA as mentioned earlier.

And the remaining cells were prepared for flow cytometric analysis to check the plasma B cell population with/without restimulation; staining and gating strategy was also followed similarly as mentioned earlier.

4-2-8. Statistical analysis

All data are reported as mean ± SD where it is applicable, and statistically significant differences were determined by the one way ANOVA, considering significant as *P* value at< 0.05. All other data were analyzed and graphically presented using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

4-3. Results and discussion

4-3-1. PST did not elicit vector-specific immune responses

Adjuvants are known to support antigen to induce antigen specific immune response, at the same time it is expected that adjuvant itself can avoid immune response against itself. However, immunogenicity against adjuvant itself is causing unwanted side effects.

It has been shown that PST is nontoxic and did not show any adverse inflammatory reaction or symptoms in mouse [8] however, the mechanism of which persistency of antigen-specific response has yet to be known. First, inflammatory cytokines, TNF-α and IL12p40, were examined in mice treated with PST or Cholera Toxin (CT) as a positive control. PST induced a minimal, if any, level of cytokines whereas CT (at 2 μg) showed significantly high levels **(Figure 4-1A, B)**. It was noting that no detectable anti-PST IgM or IgG was observed in mice treated with PST alone or PST/OVA, indicating that PST did not act as an immunogen.

The mechanism behind the lack of immunogenicity might be due to the degradable nature of PST. Fate of PST was traced after the intranasal delivery of PST conjugated with Cy5.5 by biodistribution assay. In **Figure 4-1C,** a strong signal was found in the lung region until day 3 which was faded on days 7 to 10 and no signal at day 15, reflecting the ester linkage mediated degradability of the PST. Interestingly, the signal was observed in kidney at day 3 which continued till day 10 (**Figure 4-1C**), indicating a continuous excretion of the residual part of PST which offers a safety kinetic to apply as delivery system for vaccine. The major compound of PST is its ‘polysorbitol’ backbone comprising many sorbitol chains in the structure. Sorbitol is a widely used material in food industry as sweetener and is a modified glucose molecule [9, 10]. Furthermore, the use of sorbitol, which is the major component of PST, is approved by FDA and widely used in food industry [10]. The ester-linkages have provided degradability to PST and made it non-toxic because it was reported earlier that polysorbitol transporter having ester linkages are non-toxic due to their degradable properties [12-14].

It was also described that the poly(ether ester)-based polymer having ester linkages provided biocompatibility to the polymeric system and exhibited non-immunogenicity [15]. Moreover, in another report, Zhao et al. described the double cross-linked hyaluronic acid (a natural biodegradable polymer) possessing ester linkages showed enhanced biocompatibility and non-immunogenic nature enable its use in wide range of clinical applications [16]. Altogether, it suggests that PST also showed non-immunogenic property on its own, not to stimulate adverse immune reaction due to its degradable nature by having ester linkages and ensures its safety profile as vaccine carrier.

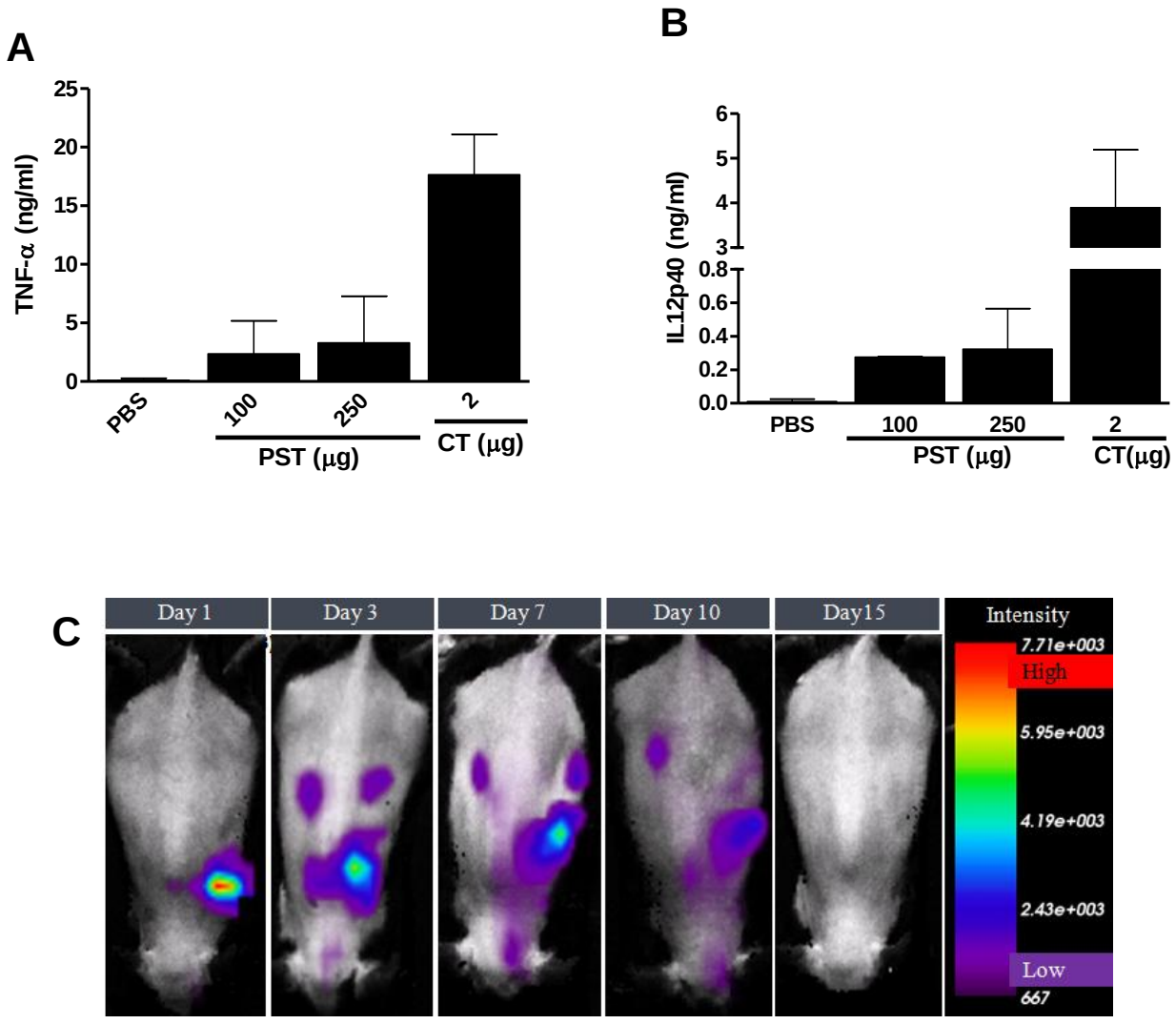


Figure 4-1. Examination of inflammatory cytokines and half-life of PST. To test immune stimulating activity of PST, C57/Bl6 mice were intranasally administered with PST (100 and 250 μg/mouse) and CT (2 μg) as a positive control. Blood samples were collected at 24 h, and the concentration of (A) TNF-α and (B) IL-12p40 was measured in serum. All data are presented as mean ± SEM. by one-way ANOVA. C) To examine the fate of PST after the intra-nasal delivery, mice were treated with Cy5.5PST/OVA complex via intranasal route. Images of anesthetized mice were captured at various time points (days 1, 3, 7, 10 and 15) using imaging system.

4-3-2. PST/OVA induced enhancement and persisted OVA-specific antibody responses

Next, I examined the OVA-specific serum antibody production. Anti-OVA IgG, IgG subtypes and IgA titers in serum and BAL fluid were significantly higher all times examined in PST/OVA group when compared to naked OVA and were well comparable to those of CT/OVA group (**Figure 4-2A-G**). To examine whether PST/OVA can induce a persistent antibody response, OVA-specific serum IgG and IgA titers were measured at days 60, 90 (**Figure 4-2D-G**) and 120 (data not shown) after the third intranasal immunization. Both IgG (**Figure 4-2D, E**) and IgA titers (**Figure 4-2F, G**) were significantly and persistently high in mice immunized with PST/OVA, showing that PST/OVA induced a long-term antigen-specific antibody response. There results suggest that the PST/OVA induced a good OVA-specific IgG responses for long period of time without PST-specific antibody response. Moreover, it is obvious that some other factors like osmotic property of PST [12-14] and adjuvant activity of PEI [17] might also augment immunity.

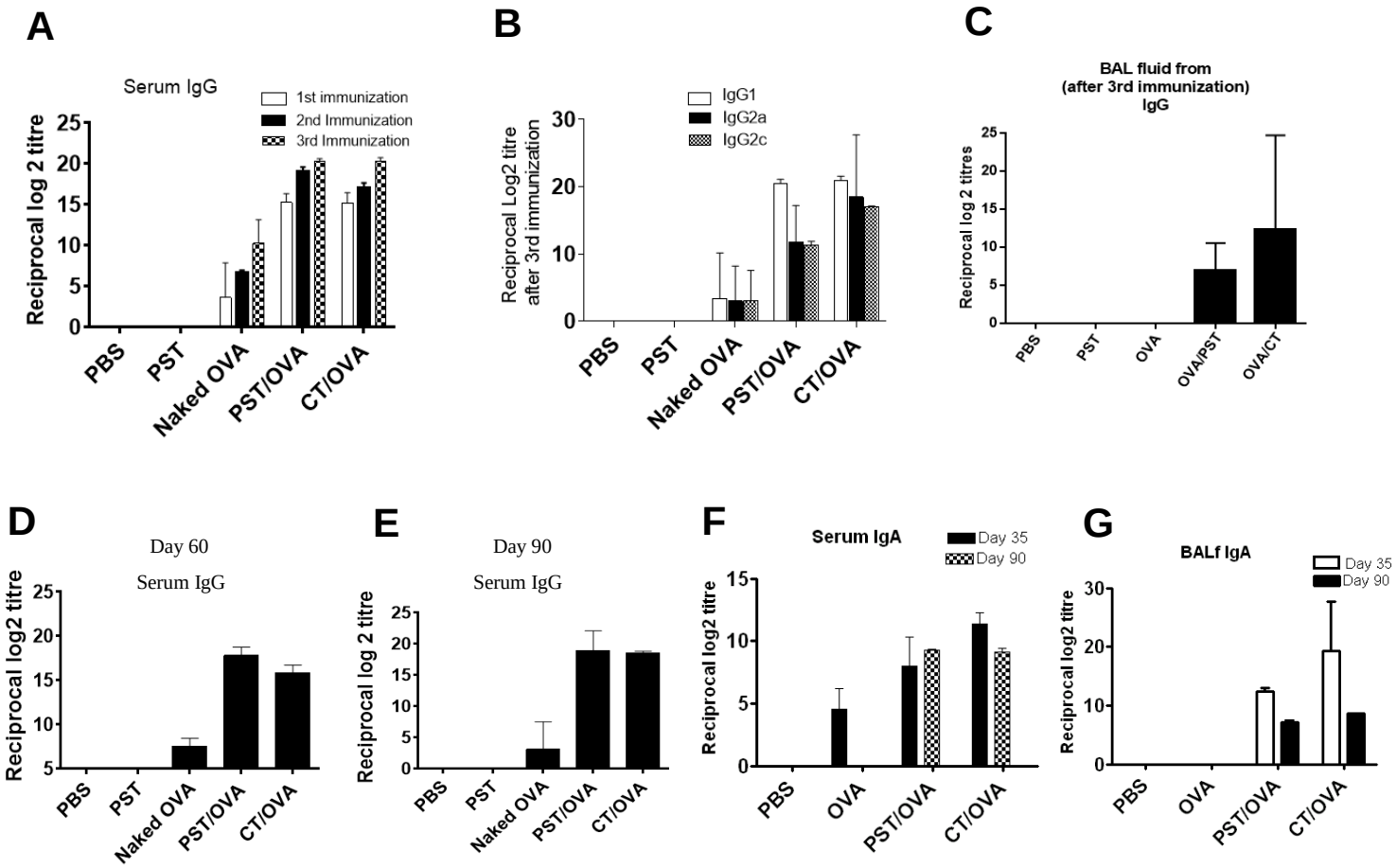


Figure 4-2. OVA-specific antibody responses in mice treated with PST/OVA or CT/OVA formulation. Mice were immunized three times with PBS, PST, OVA, PST/OVA, CT/OVA giving two weeks interval and the 3rd (the last) immunization was considered as day 0. Serum samples were collected at days -14, 0, 7, 60 and 90. Levels of OVA-specific (A) serum IgG, after 1st, 2nd, and 3rd immunization; (B) serum IgG subtypes after the 3rd immunization; (C) BAL fluid IgG after the 3rd immunization; (D, E) serum IgG at days 60 and 90, respectively; (F, G) serum and BAL fluid IgA, respectively at days 35 and 90. (Mean ± SD, n=3).

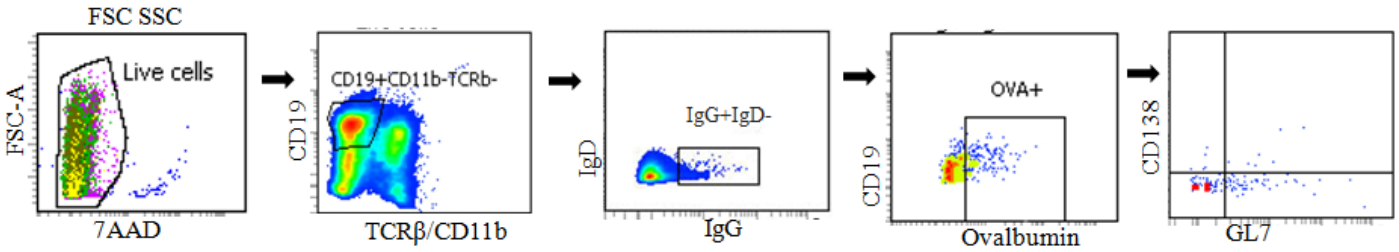
4-3-3. Characteristic of OVA-specific B cells responsible for long-lasting anti-OVA antibody response

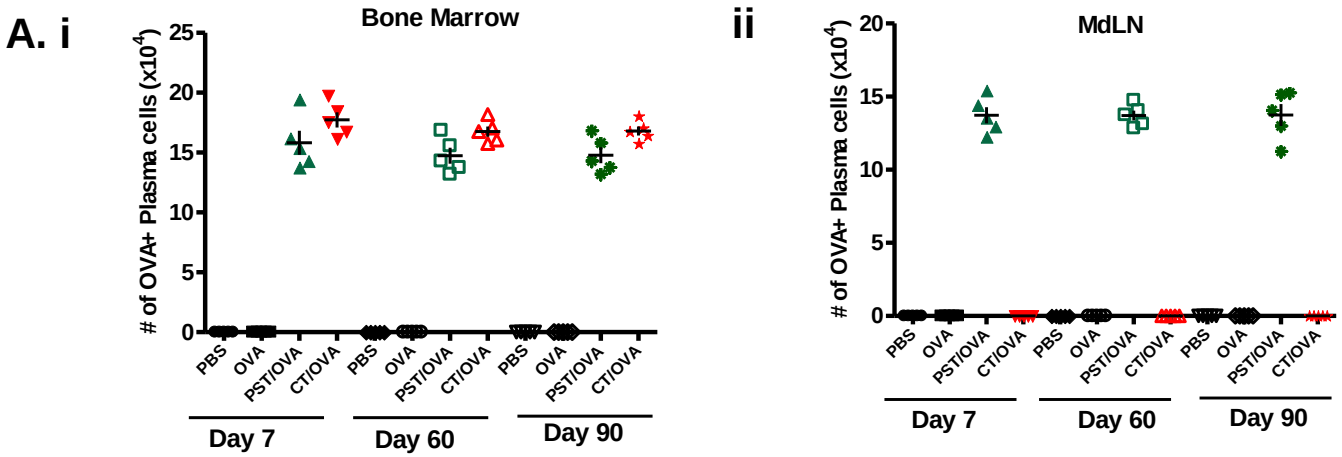
4-3-3-1. Maintenance of OVA-specific plasma cells in bone marrow and draining lymph nodes

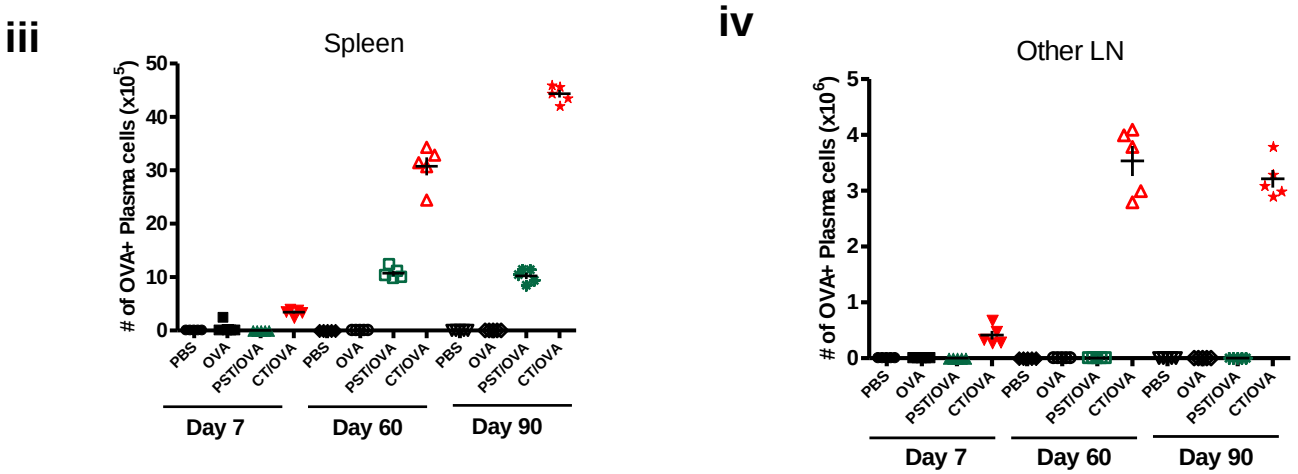
Since OVA-specific antibody response persisted for a long period of time (over 120 days) in OVA/PST group I decided to examine long-lived plasma cells responsible for the maintenance of peripheral antigen-specific antibody circulation. It was reported that these cells are migrating to bone marrow in both T cell-independent and -dependent manner at the early time after the immunization [18]. To trace the kinetics of OVA-specific plasma cells, organs were isolated at days 7, 60 and 90. In lung, both PST/OVA- and CT/OVA-treated mice showed high number of plasma cells at early time point; however, sharply declined thereafter **(Figure 4-3A i)** supporting the hypothesis for the disappearance of germinal centers in the lungs after the challenge [19]. In spleen, CT/OVA induced very high number of plasma cells until day 90 whereas PST showed no change **(Figure 4-3A ii)** suggesting the fact that the germinal centre of spleen might continuously produce and maintain a balance of OVA-specific short-lived antibody secreting cells (ASCs) or those cells reached into long survival lineage.

Therefore, it is understandable that some other possibility of reaching into long survival lineage might be involved here [20, 21] and the low level of plasma cells in spleen in PST/OVA immunized mice strongly indicates that persisting antibody response might be induced from some other major immune organs like lymph nodes specially the draining ones. Thus I sketched out the possibility that mediastinal lymph nodes (MdLNs) as a major target for intra nasal delivery and examined along with other lymph nodes (cervical and mesenteric LNs). Surprisingly, I found that PST/OVA, unlike the CT/OVA, group showed a large number of plasma cells throughout the experimental period from days 7 to 90 **(Figure 4-3A iii)**. It was noting that CT/OVA group showed the plasma cell population

in a greater number in all other lymph nodes **(Figure 4-3A iv)**. This result indicates that MdLN is playing a major role for PST-mediated OVA immunization and continued to provide a long-lasting persistent immunity might be due to sustained release of antigen from the MdLNs to the target site. Both CT and PST mediated OVA vaccination showed the presence of this cell population in the bone marrow from Days 7 to 90 as expected, indicating that the long-lived antigen-specific plasma cells were induced at the early time point and maintained in the bone marrow as well [18].


FSC SSC
Live cells
FSC-A
7AAD
CD19+CD11b-TCRb-
CD19
TCRβ/CD11b
IgG+IgD-
IgD
IgG
OVA+
CD19
Ovalbumin
CD138
GL7


A. i
Bone Marrow
# of OVA+ Plasma cells (x10^4)
PBS
OVA
PST/OVA
CT/OVA
Day 7
Day 60
Day 90
ii
MdLN
# of OVA+ Plasma cells (x10^4)
Day 7
Day 60
Day 90


iii
Spleen
# of OVA+ Plasma cells (x10^5)
Day 7
Day 60
Day 90
iv
Other LN
# of OVA+ Plasma cells (x10^6)
Day 7
Day 60
Day 90

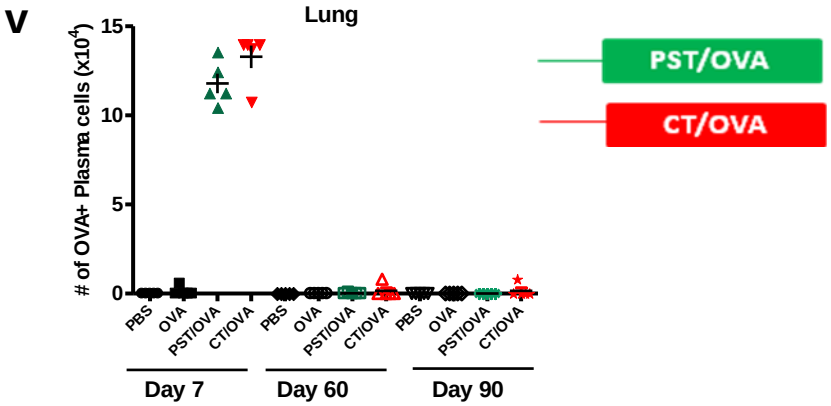

v
Lung
# of OVA+ Plasma cells (x10^4)
Day 7
Day 60
Day 90
PST/OVA
CT/OVA

Figure 4-3. Proportion of OVA-specific plasma cells at days 7, 60 and 90 post-last immunization in mice immunized with PST/OVA. Mice were immunized three times with two weeks intervals and 3rd (last) immunization was considered as day 0. Serum samples were collected at day -14, 0, 7, 60 and 90..Single cells were prepared from i) bone marrow, ii) mediastinal lymph node (MdLN), iii) spleen, iv) other LNs (Cervical+ Mesenteric), and v) bone marrow. Total numbers of TCRβ^{-}CD11b^{-} CD19^{+}IgD^{-}IgG^{+} GL7^{-}CD138^{+} OVA^{+} B cells / organ were analyzed. The PST/OVA group showed OVA-specific populations in MdLN and bone marrow **(Figure 4-3)**. This was further validated in the confocal data. Results in **Figure 4-4** showed that OVA bound on B cells are mostly in MdLN and with a few in the bone marrow, but not in lung and spleen.

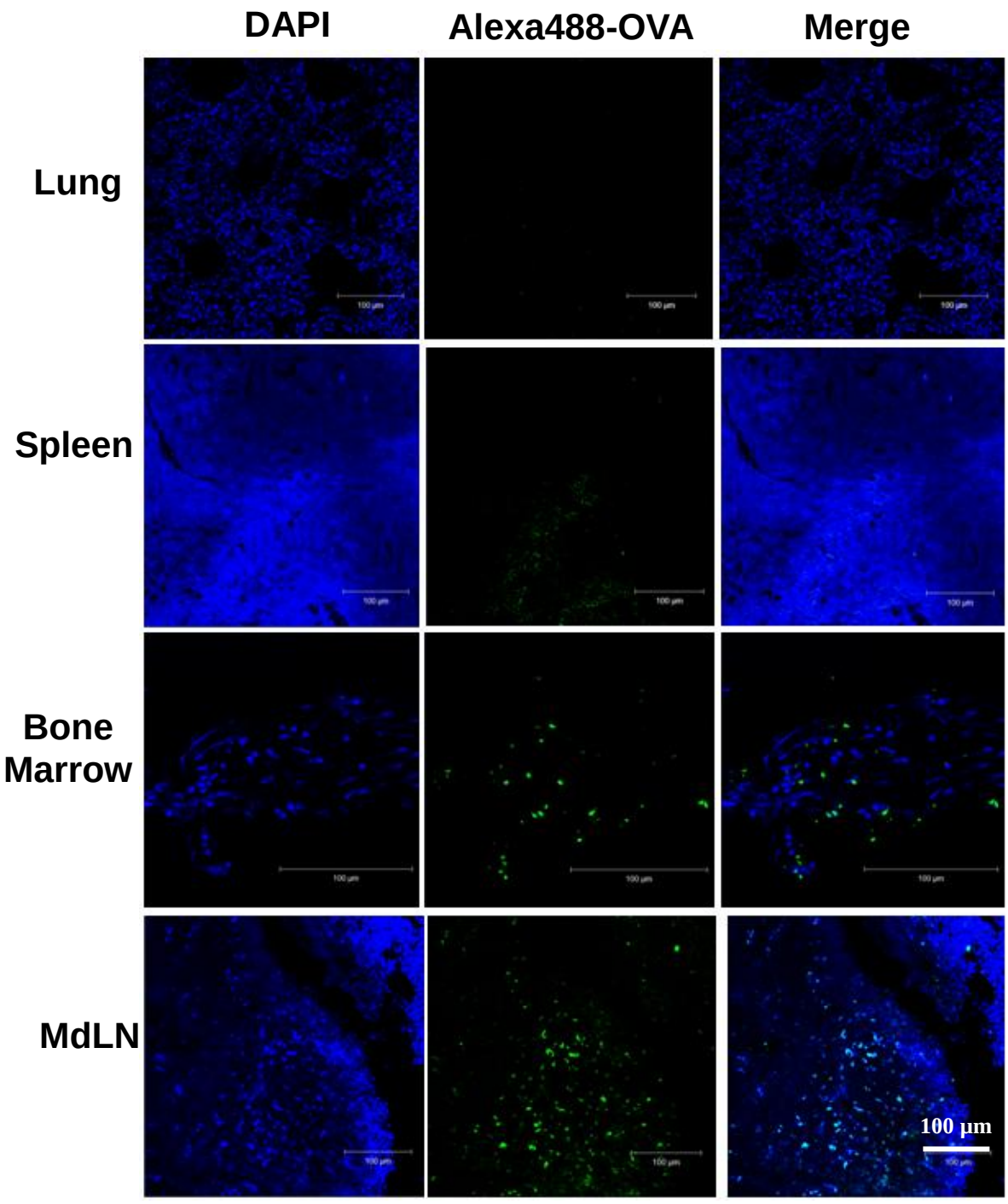

DAPI
Alexa488-OVA
Merge
Lung
Spleen
Bone Marrow
MdLN
100 μm

Figure 4-4. OVA-specific B cells at days 90 post-last immunization in mice immunized with PST/OVA. Mice were immunized with PBS, PST/OVA and at day 90 several tissue samples like lung, MdLN, Bone marrow and spleen were isolated. Tissue samples were prepared by cryosectioning, staining with Alexa-488 labeled OVA and mounted by DAPI. Confocal microscopy was used to capture sample images. Images are representative of two independent experiments with draining lymph nodes obtained from 2-3 mice per treatment. Scale bars denote 100 μm.

4-3-3-2. OVA-specific germinal center B cells effectively proliferate into antigen-specific plasma cells *ex vivo*

Germinal center (GC) is the site within the secondary lymphoid organs for proliferation, differentiation, and maturation of B cells. In **Figure 4-5A,** the absolute number of GC B cells in spleen, MdLNs and other LNs of both CT/OVA and PST/OVA groups showed plasma cell higher compared to non-restimulated population (**Figure 4-3C ii, iii and iv**).

Next, to validate the long-term persistency of PST/OVA, cells from spleen, MdLN and other lymph nodes were taken and re-stimulated with OVA *ex-vivo*. Consistent with the previous data, neither the MdLN of OVA/CT treated mice nor the other LNs of PST treated mice has shown the increase of OVA-specific plasma cells (**Figure 4-5B**).

In all cases after the OVA restimulation, the percentage of total OVA-specific plasma cells were increased, indicating that upon restimulation of same antigen effective GC B cells generated plasma B cells. Elevated OVA-specific IgG was found in all groups after the restimulation (**Figure 4-5C**). Taken together, MdLN as draining lymph node in mice immunized with PST/OVA, providing sites for OVA-specific germinal centers B cells to be maintained for long term even after 90 days, suggesting that they potentially can differentiate into plasma cells and secrete antibody upon antigen

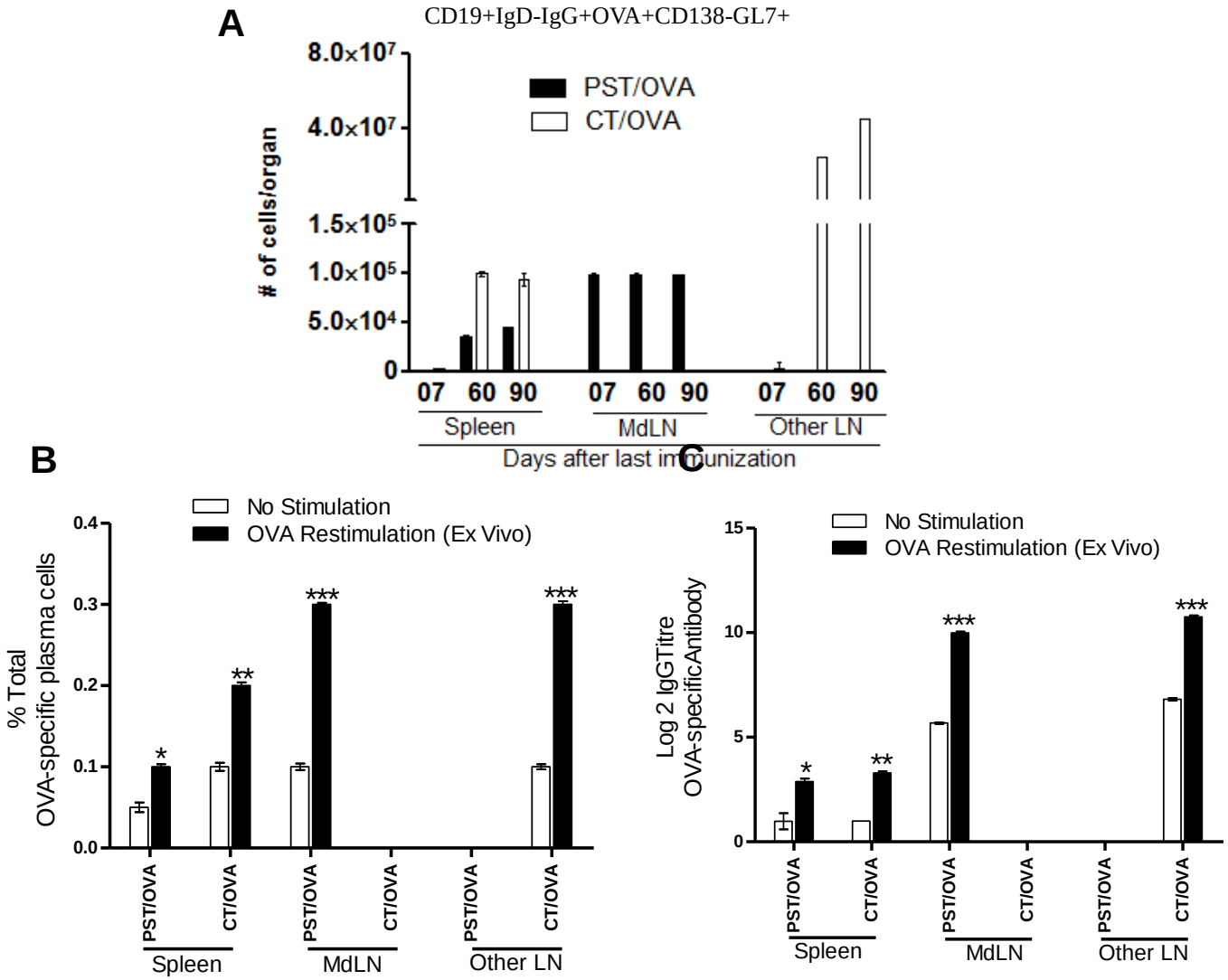


Figure 4-5. Specificity of OVA-specific germinal center B cells in mice immunized with OVA-PST up to 90 days after the last immunization. (A) Mice were immunized three times with two weeks intervals and 3rd (last) immunization was considered as day 0. Mice were sacrificed and organs were collected at day 7, 60 and 90. Single cell suspension was cultured with/with restimulation. On day 5 of *in vitro* culture cells were stained to check for Ova-specific (A) Germinal center B cells and (B) Plasma B cells by flow cytometry. Supernatant from the culture were analyzed for C) IgG level by ELISA.

exposure. It is believed that PST is able to bind to B cells and OVA which was in the complex with PST activates B cells effectively. However, when it is complexed with the OVA to B cells and then, the complex could activates and all these synergistically resulted in the development of persisted antigen-specific antibody response

4-4. Summary and Future Prospective

In summary, I have designed and successfully synthesized PST which is showing dual role as an efficient delivery tool and also as efficient adjuvant for OVA. PST exhibited stable condensing ability of OVA by simple mixing without any significant damage of OVA's functional structure. Remarkably, PST was found -incapable of showing immunogenicity *in vivo*. OVA delivered with PST showed improved antibody responses and interestingly, the antibody stimulation was persisted for a longer periods of time (>90 days after last immunization). The mechanism studies interestingly showed that PST continuously facilitated OVA-specific plasma cells and germinal centre B cells in the MdLN from the early time to the longer periods of time which probably induced by some the striking features of PST such as the safety of PST (presumably because sorbitol is a modified glucose) which helped not to produce vector-specific antibodies; and PST has facilitated the maintenance of antigen specific B cells in the draining lymph nodes that undergoes for clonal expansion to show persisted antibody response. The overall mechanism of PST is presented in the schematic **Figure 4-6**. In conclusion, results from Part II and Part III, it is indicative that PST is a promising adjuvant candidate and functional delivery material for vaccine, devoid of side effects, able to stimulate innate immune system and most importantly induce the long-lasting antibody response with some unique mechanism of stimulating the antibody secreting cells from draining lymph nodes along with bone marrow, thus seeking more investigation to use for future study.

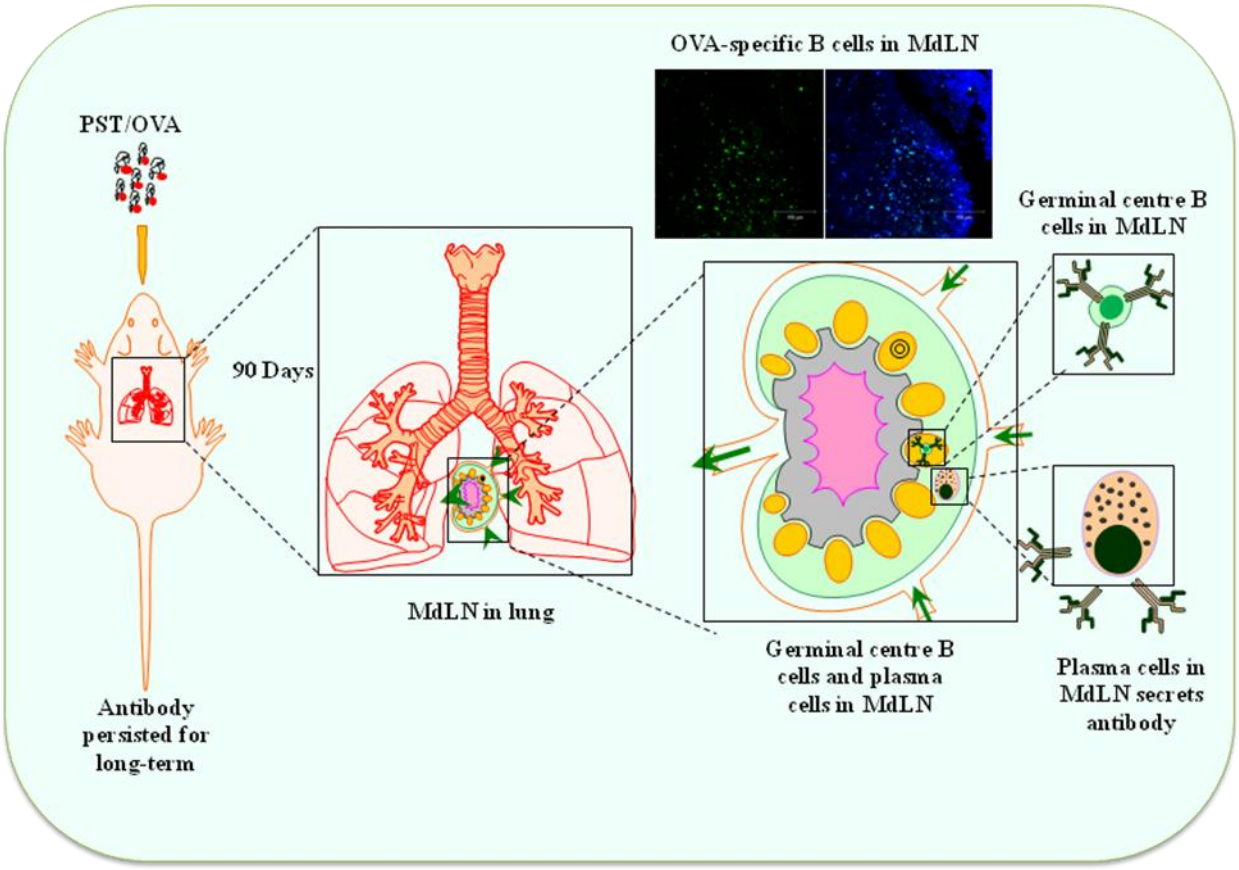


Figure 4-6. A schematic representation of the overall mechanism of PST facilitated the maintenance of long lasting antigen-specific antibody secreting cells in draining lymph node.

4-5. References

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Chapter V

Concluding Remarks

Accelerated but controlled, long-lasting and protective immunity should be the most important concern for developing novel vaccine in addition to safety profile which could provide unconditional benefits against a wide range of diseases. As a vaccine delivery system (and adjuvants in some context), nano-biotechnology and the recent advancement of material science boosted up its research field with high potential. However, we still do not have much clear pictures on the interaction of nanomaterials and the immune system which pushes backward of the field. Therefore, a few important factors should keep in mind while biomaterials are designed as carrier systems and/or adjuvants for vaccine are non-toxicity, no material-specific immunity, and the activation of innate immune system. Finally, induction of a long-lasting antibody response is one of the most important protective mechanisms for host. Wide diversity with tunable properties and recent advances in polymer chemistry and technology offers to develop effective vaccine delivery system and/or adjuvants with improved and desirable properties. In this regard, the novel polysorbitol transporter, synthesized by reaction with sorbitol molecules to crosslink with LMW PEI (MW: 600), showed high effectiveness both as a delivery system and an adjuvant for RSV glycoprotein and model protein Ovalbumin and potentially exhibited excellent ability throughout the studies to provide all the above-mentioned essential properties which is lacking in current vaccination technology.

In the first study, PST provided significant efficacy for its application both as a potential adjuvant system and polymeric delivery tool for respiratory syncytial virus (RSV) glycoprotein antigen. Respiratory syncytial virus (RSV) is one of the most common causes of viral deaths in infants worldwide, yet there are no effective vaccines available. PST showed a potent, yet safe, adjuvant activity and acting as an effective delivery tool for RSV glycoprotein (RGp) antigen. The safety profile of PST was excellent that showed no toxicity *in vitro* and *in vivo* unlike PEI

and the pre-existing experimental mucosal adjuvant, cholera toxin (CT). The osmotically active PST formed nano-sized complexes with RGp by simple mixing, retained the antigenic stability and exhibited negative surface charges made them highly effective for the enhancement of phagocytosis-mediated uptake. This resulted in improved cytokine expression and the significant augmentation of RGp-specific antibody production with long-term persistency over 200 days. Interestingly, PST/RGp enhanced phagocytic cell-mediated uptake owing to the osmotic property of PST and negative zeta potential, suggesting that PST mediated phagocytic cell stimulation could be one of the reasons for the long-lived antigen-specific antibody response.

In the second study, the action mechanism of naked OVA and PST/OVA is compared at the early time of administration when the naked OVA is reported to be degraded. The results showed that PST/OVA targeted cells are mostly APCs, especially in MdLN, which provided a platform for iNKT cell activation at germinal center in response to higher CD1d expression by macrophage, dendritic cells, B cells and neutrophils. Taken together, these results showed that PST-mediated OVA delivery induced increased number of CD1d positive phagocytes and antigen presenting cells potentially responsible for robust and long-lasting immune response via iNKT cell activation. The possible mechanism of inducing CD1d expression might be occurred due to the sorbitol part of PST which is a modified sugar molecule and it was reported that the CD1d grove is the site for binding of the sugar moiety of different lipid/polysaccharide antigen.

The third study attempts to reveal the distribution of PST in relation to how it induced long-lasting antibody response. Remarkably, PST was found non-immunogenic on its own *in vivo*, thus able to avoid the risk of vector-specific antibody response which is considered as the prime concern in developing effective vaccine delivery system and adjuvant. OVA delivered with PST showed improved OVA-specific antibody responses and supported the proof-of-concept of the

previous finding that the antigen-specific antibody response can be persisted for a long period of time. Interestingly, the mechanism studies showed that PST caused long-term maintenance of OVA-specific plasma cells and germinal center B cells in the mediastinal lymph node (MdLN) along with bone marrow which might be the reason behind the long-lasting antibody production.

Collectively, I have successfully synthesized and executed PST which showed a dual role as an efficient delivery tool and as an adjuvant. A numerous results from various approaches indicate that PST could be a promising adjuvant and effective delivery strategy for vaccine, devoid of side effects, able to stimulate innate immune system. Most importantly it induces the long-lasting antibody response with the via showing the higher number of CD1d expressing cells (**Figure 5-1**) of stimulating the antibody secreting cells from draining lymph nodes as well as bone marrow.

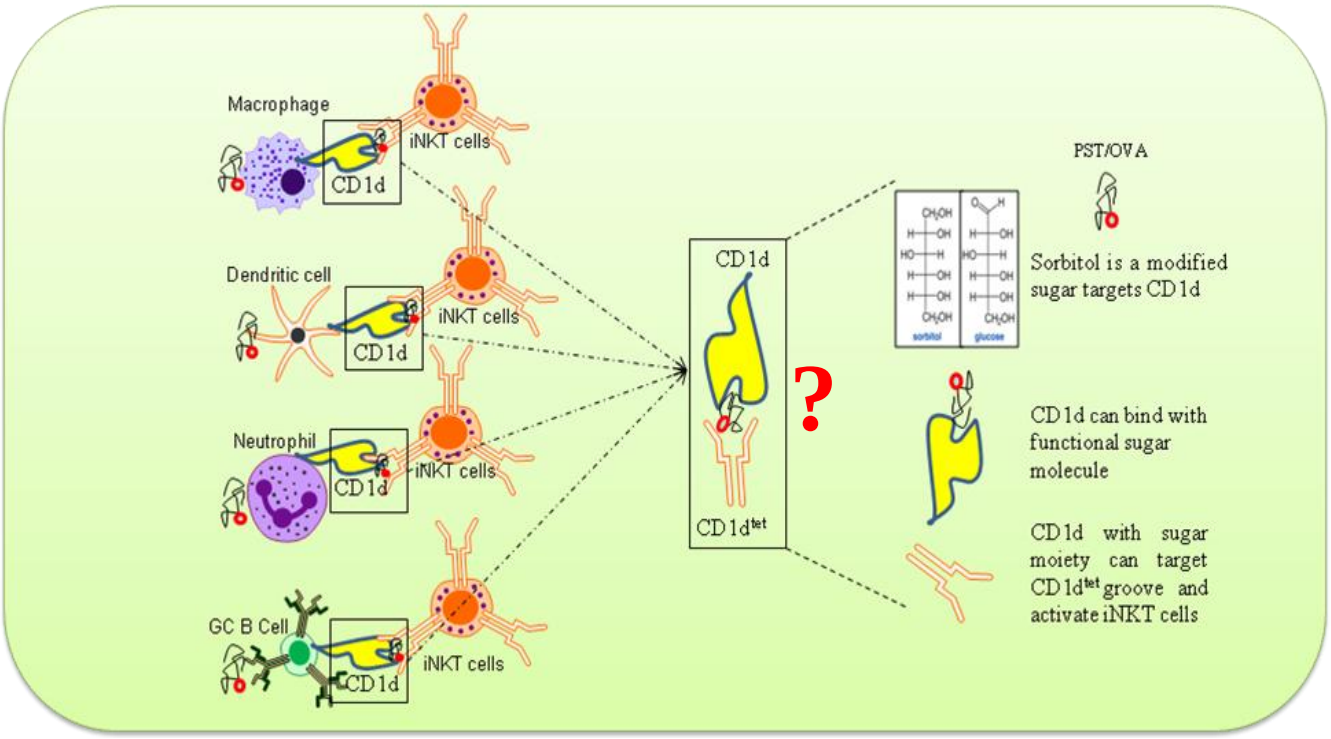

Figure 5-1. A possible unknown mechanism of CD1d mediated iNKT cells activation.

List of Publications

Published

1. **Firdous J**, Islam MA, Park SM, Cheon IS, Shim BS, Yoon HS, Song M, Chang J, Choi YJ, Park YM, Boraschi D, Han SH, Cho CS, Yun CH. Induction of long-term immunity against respiratory syncytial virus glycoprotein by an osmotic polymeric nanocarrier. Acta Biomater. 2014 Nov; 10(11):4606-17. [**IF 5.608**].

2. Islam MA, Park TE, Singh B, Maharjan S, **Firdous J**, Cho MH, Kang SK, Yun CH, Choi YJ, Cho CS. Major degradable polycations as carriers for DNA and siRNA. J Control Release. 2014 Nov 10; 193C:74-89. [**IF 7.261**].

3. Islam MA, **Firdous J**, Choi YJ, Yun CH, Cho CS. Regulation of endocytosis by non-viral vectors for efficient gene activity. J Biomed Nanotechnol. 2014, January; 10 (1): 67-80. [**IF 7.578**].

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5. Islam MA*, **Firdous J***, Yun CH, Cho CS. Design and application of chitosan microspheres as a potent oral and nasal vaccine carrier. Int J Nanomedicine. 2012 Dec; 7: 6077-6093. [**IF 3.463**] [***Equally contributed as 1st author**].

Book Chapter

1. Ju YJ, **Firdous J**, Yoon HS, Han SH and Yun CH, Autophagy: Principles, Regulation and Roles in Disease. Chapter: Cross-presentation induced by autophagy towards clinical approaches. 2012; Nova Science Publishers, Inc. Hauppauge NY 11788-3619, USA.

Submitted/or under preparation

1. Islam MA, Park TE, **Firdous J**, Choi YJ, Yun CH, Cho CS. Design of polymeric gene carriers for highly efficient DNA transfection and RNAi silencing through regulation of cellular uptake. Chem. Soc. Rev. 2014 **[IF 30.425]**.
2. Interaction of polysorbitol transporter with B cells of draining lymph node and enhanced CD1d expression in APCs at early time lead to long-lasting antibody response **[Manuscript under preparation]**.
3. Polysorbitol transporter facilitates the maintenance of long-lasting antigen-specific potent germinal centre B cells in draining lymph node **[Manuscript under preparation]**.

Awards/Honors:

Year	Name of Honor/Prize	Awarding Organization	Achievement for which Awarded
2013	WCU Best Poster Award	World Class University (WCU) Biomodulation Department at Seoul National University, South Korea	The award was given under PhD research excellence category in 2013
2013	Han's Animal Life Science Award	Han's Animal Life Science Foundation (HALSF)	For excellent research activity in 2013